CALCIUM HOMEOSTASIS IN LENS TRANSPARENCY
AND
THE INVOLVEMENT OF CALPAINS IN CATARACT

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

At
Lincoln University
By
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Lincoln University
2006
Abstract of a thesis submitted in partial fulfillment of
the requirements for the Degree of Ph.D.

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by Hannah Yun Young Lee

Purpose. The absolute clarity of the lens of the eye is vital in the visual system. The unique structural and physiological properties of the lens are tightly integrated with highly ordered protein content to allow the lens to remain transparent. Consequently, any alteration or disturbance of these highly ordered proteins can affect the optical properties of the lens. In humans, cataracts are the major cause of blindness, yet the exact aetiology of cataract formation (cataractogenesis) is not fully understood. The purpose of the current research was to investigate whether deregulation of the Ca$^{2+}$-dependent enzyme, calpains, following changes in lens Ca$^{2+}$ homeostasis, is a key mechanism leading to undesired cleavage of a number of proteins that are linked with maintaining lens transparency and contributing to cataractogenesis.

Methods. An ovine lens culture (in vitro) system and the heritable ovine cataract (in vivo) model were used to test the research hypothesis. The Ca$^{2+}$ ionophore, ionomycin, was used to induce a Ca$^{2+}$ overload and in vitro opacification during lens culture. Opacity in the lens was graded by a computer image analysis program. Protein profile (SDS-PAGE, 2-DE and Western detection), calpain activity (casein zymography), lens structure (microscopic view) and cytotoxicity level (LDH leakage assay) were analysed in Ca$^{2+}$-induced opaque lenses. The involvement of calpain during opacification was further examined by applying synthetic exogenous calpain inhibitors to the in vitro system. Two novel exogenous calpain inhibitors were also assessed for their therapeutic potential in preventing the progression of cataracts in the in vivo cataract model by topical administration of the inhibitor direct to the sheep's eye over a 11 week period. HPLC was used to detect the penetration of these compounds into ocular tissues.
Results. Sustained Ca$^{2+}$ influx into cultured lenses caused dense opacification. The opacity was characterised by formation of a turbid fraction and cell death in the outer cortex of the ovine lens. There was increased calpain autolysis associated with the progress of opacification, indicating increased calpain activity. Major degradation of the cytoskeletal proteins, spectrin and vimentin, was observed whilst there was limited degradation of the lens structural soluble proteins, crystallins, in response to a Ca$^{2+}$ flux. Lens proteins were protected from degradation by adding synthetic calpain inhibitors to the culture medium. Topical administration of novel anti-calpain molecules failed to retard the progression of cataractogenesis in the ovine inherited cataract model. Further investigation of drug penetration showed that efficacy of inhibitory compounds was limited by permeability of these molecules across the cornea and the ability of the molecules to reach and penetrate into the lens.

Conclusion. The ovine lens Ca$^{2+}$-induced opacification (OLCO) model in this thesis has provided a model to understand the role of Ca$^{2+}$ homeostasis in lens transparency. With sustained intracellular Ca$^{2+}$ level, the degradation of cytoskeletal elements is highly correlated with calpain activity. Cataractogenesis is the pathological response to the loss of lens Ca$^{2+}$ homeostasis in this model. The current results support the hypothesis that the deregulation of calpain activity is an trigger for a series of cascading events, leading to death of the cells in the lens.

Keywords: Cataractogenesis, Ca$^{2+}$ homeostasis, proteolysis, calpain, cytoskeletal proteins, crystallins, calpain inhibitors, ovine lens, culture system, OLCO model, cell death and the inheritable ovine cataract model.
Acknowledgements

I would like firstly to express my sincere and grateful appreciation to my supervisor Dr Jim Morton for his generous assistance, continued support, encouragement and guidance throughout the course of my work and in the development of this thesis. Thank you Jim for giving me the opportunity to work in the Cataract team and for your efforts in assisting me to secure an Enterprise scholarship. I would also like to express my sincere gratitude to my associate supervisor, Professor Roy Bickerstaffe, for his critique of my thesis draft and invaluable advice throughout the preparation of my thesis. Special thanks also to Dr Lucinda Robertson for providing technical advice, assisting in collecting samples, and sharing her extensive knowledge throughout my research years.

I would like to thank additionally all past and present fellows of the Lincoln University Cataract team; Dr Graham Kay, Karl Gately, Matthew Muir, Jie Lie, Josh McDermott, Anita Hancock, Tony Dennison and Gareth Wilson. I would particularly like to thank my fellow PhD student Matthew Muir, staff technician Karl Gately and Dr Lucinda Robertson, who have always been readily available and keen to lend their valuable time to help me out, especially taking care of 'the trial business' during my short absence. My thanks to Karl, our generous caring 'cheque' man, for ensuring the smooth running of the lab, making it as friendly as possible. Thanks too to fellow Masters students Jie and Josh for sharing their expertise towards the current work.

I am very grateful to the faculty and staff at Lincoln University and Malvern Abattoir for their generous assistance throughout the years. I would like to thank Nigel Jay (JML) for providing assistance on running in vivo trials, and Dr Steve Heap (Veterinarian) for examining the lambs' eyes for cataracts. Thanks also to Jenny Zhao and Lynne Clucas for their technical advice and help with the analysis. To my fellow PhD Li Hong, thank you for your incredible wit. I would also thank Professor Andrew Abell, Professor Jim Coxon and their cataract team at the University of Canterbury for supplying the inhibitor compounds.

I also owe a great deal of thanks to a number of overseas collaborators; Dr Larry David and his Laboratory team and Professor Tom Shearer at OHSU in Portland, Dr Mitsuyoshi Azuma and Dr Emi Nakajima and other Senju Laboratory members in Portland. I would also express
my appreciation to Dr Julie Sanderson for sharing invaluable knowledge of sheep lens culture, which has been the foundation of my thesis.

I would also like to acknowledge with gratitude the Foundation for Research and Science and Technology (FRST) Enterprise Scholarship (2002-2005), Douglas Pharmaceuticals Limited, the Canterbury Branch of the Federation of University Women Trust (2004), the New Zealand Society for Biochemistry and Molecular Biology (2004) and Lincoln University – Dr Jim Morton Cataract Group (2003 and 2004), for their financial support throughout my studies.

Lastly I would like to express my deepest appreciation to all friends in New Zealand and other parts of world for the support and companionship they have shown during the good, bad, stressful, happy and tough times of my PhD years. Special thanks to Melissa, Huia and Jong Hee, who have inspired me with their true strength.

Finally, I would share the joy of achievement with my family in Korea, mum, dear brother Sang Hoon and my grand parents.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xvi</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xvii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>xix</td>
</tr>
</tbody>
</table>

Chapter 1: Literature Review

1.1. Vision and Cataract 1

1.2. Eye 2

1.3. Lens in Eye 3

   *Epithelium and fiber cells of the lens* 4

1.4. Components for the Transparent Lens 5

   *Lens soluble structural proteins, crystallins* 5

   *Lens cytoskeletal elements* 7

   *Elimination of lens organelles* 9

1.5. Microcirculation 10

1.6. Physiological Zones in the Lens 12

1.7. Ca$^{2+}$ Regulation 14

   *Ionomycin* 16

1.8. Lens Ca$^{2+}$ Homeostasis 16

1.9. Ca$^{2+}$ Homeostasis and Cataract 18

1.10. The Calpain Proteolytic System 19

vi
Chapter 2: Experimental Rationale
2.1. Introduction
2.2. Hypothesis for Current Research
2.3. Research Methods: Ovine Lens Models
2.4. Outlines of Research Chapters
2.5. Outline of General Conclusion Chapter
Chapter 3: Viability of the Intact Ovine Lens in Culture System
3.1. Introduction
3.2. Methods
   Preparation of culture medium
   Lens sample collection
   Experimental protocol for lens viability with two culture media
   Experimental protocol for lens viability with pre-EMEM treatment
   Visual monitoring of cultured lenses
   Lens opacity grade scoring using an image analysis system
Chapter 4: Ionomycin Induced Opacity in Ovine Lens Culture

4.1. Introduction

4.2. Experiment 1: Ionomycin induced opacification in intact ovine lens

4.2.1. Methods

Preparation of treatment solutions

Lens culture

Experimental protocol

Colorimetric calcium assay

Analysis of lens proteins

Casein gel zymography

SDS-PAGE and Immunoblotting (Western detection)

Two-dimensional electrophoresis (2-DE) analysis

4.3.2. Results

4.4. Experiment 2: Progression of opacity in intact ovine lens by ionomycin

4.4.1. Methods

Lens sample collection and culture preparation

Experimental protocol

4.4.2. Results

4.5. Experiment 3: Change of calpain II activity levels

4.5.1. Methods

Ovine lens collection and the culture preparation

Experiment protocol
Calpain activity analysis by casein zymography 63

4.5.2. Results 63

4.6. Experiment 4: Cytotoxicity and morphological changes in the OLCO model. 64

4.6.1. Methods 64

*Lens sample collection & culture preparation* 64

*Preparation of treatment solution* 64

*Experimental protocol* 65

*Ca\(^{2+}\) assay by atomic absorption analysis* 65

*Cell viability assay with cytotoxicity detection kit.* 65

*Microscopic view of ionomycin-treated lenses* 65

*Lens opacity grade scoring by image analysis system* 66

4.6.2. Results 66

4.7. Discussion 71

4.8. Conclusion 80

Chapter 5: Application of Calpain Inhibitors to Cataractogenesis Models 81

5.1. Introduction 81

5.2. Experiment 1: Application of calpain inhibitor in the culture system. 83

5.2.1 Experiment 1-a: Calpain inhibitor in the OLCO model 83

5.2.1.1 Methods 83

*Lens culture* 83

*Preparation of treatment solutions* 83

*Experimental protocol* 83

*Grading of opacification* 84

*Analysis of calpain activities and protein profiles* 84

5.2.1.2. Results 84
5.2.2. Experiment 1-b: Calpain inhibitor in the hyper-Ca$^{2+}$ medium model

5.2.1.2. Methods

*Lens culture*

*Preparation of treatment solutions*

*Experimental protocol*

*Analysis of calpain activity and protein profiles.*

5.2.2.2. Results

5.2.3. Discussion

5.3. Experiment 2: Assessment of novel inhibitors in vitro and in vivo

5.3.1. Novel calpain inhibitors in in vitro culture system

5.3.1.1. Methods

*Lens culture*

*Preparation of treatment solution*

*Experimental protocol*

*Visual image analysis*

5.3.1.2. Results

5.3.2. Topical application of novel calpain inhibitors to in vivo cataract model

5.3.2.1 Methods

*Ethics approvals*

*Preparation of two eye-drops*

*Pre-treatment test for eye irritation with Cat0059 and 4583 eye-drops*

*Classification (scoring) for in vivo ovine cataract*

*Selection of lambs for inhibitor trial treatments*

*Protocol for eye-drop treatments*

*Sample collections*

*Extraction of calpain inhibitors from dissected samples*
Determination of UV spectrum of inhibitors

Measurement of extracted samples by HPLC

5.3.2.2. Results

5.3.3. Discussion

Chapter 6: Drug Profile of the Novel Calpain Inhibitor, Cat0059

6.1. Introduction

6.2. Experiment 1: Corneal permeability of Cat0059

6.2.1 Methods

Eyeball preparation

Experimental protocol

Preparation of samples for HPLC analysis

6.2.2. Results

6.3. Experiment 2: Cat0059 in a lens culture system

6.3.1 Methods

Lens culture

Preparation of treatment solution

Experimental Protocol

Cytotoxicity of Cat0059; LDH Leakage Assay

Stability of Cat0059 in the culture condition

Uptake of Cat0059 in the lens

Measurement of the Cat0059 by HPLC

6.3.2 Results

6.4. Experiment 3: Bioavailability of Cat0059 in an ocular environment.

6.4.1 Methods
Aqueous humour and lens homogenate sample preparation
Experimental protocol for Cat0059 binding factors
Centrifugal ultrafiltration
Non-specific binding of Cat0059 to centrifugal ultrafiltration apparatus
Evaluation of extraction procedure by HPLC
Measurement of Cat0059 by HPLC analysis

6.4.2 Results

6.5. Experiment 4: Intravitreal injection of Cat0059

6.5.1. Methods

Animal sample
Formulation of 0.1% Cat0059 for intravitreal injection
Sample collection
HPLC measurement of Cat0059

6.5.2. Results

6.6. Discussion

Chapter 7: Overall Conclusion and Future Directions

7.1. Overall Conclusion

7.2. A model for Cataractogenesis:

7.3. Future Directions

REFERENCES

PUBLICATION & CONFERENCE POSTER PRESENTATIONS ARISING FROM THIS THESIS
List of Figures

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Cross-section of a mammalian eye showing the position of the lens.</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic drawing of cross section of the adult human lens (A) and arrangement of lens fiber cells and sutures in human lens (B).</td>
<td>4</td>
</tr>
<tr>
<td>1.3</td>
<td>Diagram of internal ionic current flow around and through normal lens (A) and Na⁺ transport in the lens (B).</td>
<td>11</td>
</tr>
<tr>
<td>1.4</td>
<td>Schematic drawing of lens morphology showing equatorial and axial sectioning planes with the dispersion of fiber cell nuclei (A) and diagram of ideal construction of the degree of coupling between fiber cells (B).</td>
<td>13</td>
</tr>
<tr>
<td>1.5</td>
<td>X-ray crystal structure of Ca²⁺-free (inactive) human calpain II.</td>
<td>20</td>
</tr>
<tr>
<td>1.6</td>
<td>Calpain II activation in vivo with the presence of Ca²⁺ and lipid bodies or membrane.</td>
<td>21</td>
</tr>
<tr>
<td>2.1</td>
<td>A modified diagram of the changes seen in human cataract.</td>
<td>32</td>
</tr>
<tr>
<td>3.1</td>
<td>Digital images of three ovine lenses in the culture medium on a grid including their opacification scores (or relative transparency scores) based on digital visual analysis.</td>
<td>41</td>
</tr>
<tr>
<td>3.2</td>
<td>Digital images of ovine lenses and their opacification scores, after culture for 5 h or 26 h in EMEM, EMEM + 2% Triton X-100, AAH and AAH + 2% Triton X-100.</td>
<td>42</td>
</tr>
<tr>
<td>3.3</td>
<td>A plot of LDH leakage levels released from the lenses during culturing in AAH (A) and in EMEM (B) for a 24 h period.</td>
<td>43</td>
</tr>
<tr>
<td>3.4</td>
<td>A plot of relative cytotoxicity (%) of normal ovine lens during culturing in AAH (A) and in EMEM (B) for 24 h.</td>
<td>44</td>
</tr>
<tr>
<td>4.1</td>
<td>Structure of Ca²⁺ ionophore, ionomycin, with 1:1 complex with a Ca²⁺.</td>
<td>49</td>
</tr>
<tr>
<td>4.2</td>
<td>Digital images of ovine lenses after culture in EMEM-3.6, EMEM-3.6 + 5 µM ionomycin and EMEM-3.6 + 5 µM ionomycin + 5 mM EGTA for 48 h.</td>
<td>54</td>
</tr>
<tr>
<td>4.3</td>
<td>Casein zymography of calpains extracted from ovine lung and from normal ovine lenses cultured for 72 h in EMEM.</td>
<td>56</td>
</tr>
<tr>
<td>4.4</td>
<td>Casein zymography of calpains present in 100 µg of soluble protein from lenses after culture in EMEM-3.6, EMEM-3.6 + 5 µM ionomycin and EMEM-3.6 + 5 µM ionomycin + 5 mM EGTA for 48 h.</td>
<td>56</td>
</tr>
</tbody>
</table>
Figure 4.5. Casein zymography of calpains present in 25 μg of soluble protein from lenses after culture in EMEM-3.6, EMEM-3.6 + 5 μM ionomycin and EMEM-3.6 + 5 μM ionomycin + 5 mM EGTA for 48 h.

Figure 4.6. SDS-PAGE analysis of the urea soluble protein from ovine lenses after culture in EMEM-3.6, EMEM-3.6 + 5 μM ionomycin and EMEM-3.6 + 5 μM ionomycin + 5 mM EGTA for 48 h.

Figure 4.7. Western detection of urea soluble vimentin from ovine lens proteins after culture in EMEM-3.6, EMEM-3.6 + 5 μM ionomycin and EMEM-3.6 + 5 μM ionomycin + 5 mM EGTA for 48 h.

Figure 4.8. 2-DE soluble protein profiles from ovine lenses after culture in EMEM, EMEM + 5 μM ionomycin and EMEM + 5 μM ionomycin + 5 mM EGTA for 48 h.

Figure 4.9. Digital images of an ovine lens, displaying the progression of opacification development over a 7 days culturing in EMEM with 1 μM ionomycin.

Figure 4.10. Casein zymography of calpains present in 25 μg of soluble proteins from the cortex of the ovine lens harvested at 24, 44 and 68 h after culture in EMEM with 1 μM ionomycin.

Figure 4.11. A plot of mean LDH leakage levels released from the lenses during culturing in EMEM, EMEM + 5 μM ionomycin, EMEM-3.6 + 5 μM ionomycin and EMEM + 5 μM ionomycin + 5 mM EGTA for 44 h.

Figure 4.12. A plot of mean opacification scores (%) of lenses cultured in EMEM, EMEM + 5 μM ionomycin, EMEM-3.6 + 5 μM ionomycin and EMEM + 5 μM ionomycin + 5 mM EGTA for 44 h.

Figure 4.13. Microscopic views of ovine lenses after culture for 44 h in EMEM, EMEM-3.6 + 5 μM ionomycin and EMEM-3.6 + 5 μM ionomycin + 5 mM EGTA.

Figure 5.1. Digital images of ovine lenses taken at three different times (t = 0, 48 and 96 h) during culture in EMEM, EMEM + 5 μM ionomycin and EMEM + 5 μM ionomycin + 100 μM SJA6017.

Figure 5.2. Casein zymography of calpains present in soluble protein (A) and SDS-PAGE of the urea soluble protein (B) from ovine lenses after culture for 96 h in EMEM, EMEM + 5 μM ionomycin and EMEM + 5 μM ionomycin + 100 μM SJA6017.

Figure 5.3. Digital images and opacification scores of ovine lenses after culture for 24 h experimental period in EMEM, EMEM + 5 mM Ca²⁺ and in EMEM + 5 mM Ca²⁺ + 0.8 μM SJA6017.
Figure 5.4. SDS-PAGE analysis of urea soluble proteins (A) and Western detection of urea soluble α-spectrin from ovine lens proteins in cortical region (B), after culture for 24 h in EMEM + 5 mM Ca²⁺ and EMEM + 5 mM Ca²⁺ + 0.8 μM SJA6017.

Figure 5.5. 2-DE protein profile of soluble protein extracted from the cortex fraction of the lenses after culture for 24 h in EMEM, EMEM containing 5 mM Ca²⁺, and in EMEM containing 5 mM Ca²⁺ and 0.8 μM SJA6017.

Figure 5.6. The structure of the dipeptide calpain inhibitor, SJA6017 (N-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal).

Figure 5.7. Digital image of ovine lenses and mean opacification scores of paired ovine lenses after culture in EMEM + 5 mM Ca²⁺ and EMEM + 5 mM Ca²⁺ + 0.8 μM two novel inhibitors (Cat0059 or 4583).

Figure 5.8. Progression of the ovine inherited cataract and cataract scoring system developed for inherited ovine cataract using ophthalmoscopy and slit microscopy.

Figure 5.9. A plot of mean cataract scores for untreated and treated eyes with calpain inhibitors, Cat0059 (A) and 4583 (B), during a 67 day experiment period.

Figure 5.10. A plot of mean cataract scores for untreated eyes over 67 days.

Figure 5.11. Diagram of UV spectrum scanning result of 2, 4 and 6 μg/mL Cat0059.

Figure 5.12. Chromatogram of Cat0059 (50 ng in 5 μL injection) at 300 nm UV detection.

Figure 5.13. Diagram of UV spectrum scanning results of 2, 4, 6 and 10 μg/mL 4583.

Figure 5.14. Chromatogram of 4583 (50 ng in 5 μL injection) at 329 nm UV detection.

Figure 6.1. Standard curve of Cat0059 (0-1500 ng).

Figure 6.2. A plot of mean LDH leakage levels from ovine lenses during culturing in AAH and AAH + 10 μM Cat0059, for 5.5 h.

Figure 6.3. Digital images and mean opacification scores of ovine lenses after culture in EMEM, EMEM + 5 mM Ca²⁺ and EMEM + 5 mM Ca²⁺ + 10 μM Cat0059 for a 44 h period.

Figure 6.4. Standard curve of Cat0059 (0-100 ng).

Figure 7.1. A proposed diagram of cataractogenesis in response to changes in Ca²⁺ homeostasis.
List of Tables

Table 1.1. Examples of mammalian proteins triggered by Ca\(^{2+}\). 15

Table 1.2. Characterising six domains of ubiquitous calpain I and calpain II. 22

Table 4.1. Mean wet weight (g), mean dry weight (g) and mean Ca\(^{2+}\) concentration of lenses after culture for 48 h in EMEM-3.6, EMEM-3.6 + 5 μM ionomycin and EMEM-3.6 + 5 μM ionomycin + 5 mM EGTA. 55

Table 4.2. Mean protein concentration (mg/g) of whole lenses measured after culture for 48 h in EMEM-3.6, EMEM-3.6 + 5 μM ionomycin and EMEM-3.6 + 5 μM ionomycin + 5 mM EGTA. 58

Table 4.3. Mean total ion concentrations and wet weights of whole lenses after culture in EMEM, in EMEM + 5 μM ionomycin (Iono-1.8) and in EMEM + 5 μM ionomycin + 5 mM EGTA. 68

Table 4.4 Mean total Ca\(^{2+}\) concentration (mM) in two separated regions of the ovine lens after culture in EMEM + 5 μM ionomycin (Iono-1.8) and EMEM + 5 μM ionomycin + 1.8 mM Ca\(^{2+}\) (Iono-3.6) for 2 days. 69

Table 5.1. Mean wet weight (g) of ovine lenses after culture for 96 h in EMEM, EMEM + 5 μM ionomycin and EMEM + 5 μM ionomycin + 100 μM SJA6017. 85

Table 5.2. Mean soluble protein and urea soluble protein concentrations (mg/g) extracted from the cortex region of ovine lenses after culture in EMEM + 5 mM Ca\(^{2+}\) and EMEM + 5 mM Ca\(^{2+}\) + 0.8 μM SJA6017 for 24 h. 89

Table 6.1. Measurement of Cat0059 concentration by HPLC after the cornea of the ovine globe was in contact with 3 mM Cat0059 eye-drop formula for three different durations (t = 1 h, 2 h and 3 h). 114

Table 6.2. Estimated amount (ng) of Cat0059 remaining in the culture medium at the end of each 100 min and 5.5 h incubation. 118

Table 6.3. Portion of Cat0059 bound to pooled ovine aqueous humour (%), containing 1.25 mg/mL protein. 121

Table 6.4. Portion of Cat0059 bound to pooled ovine lens homogenates (%), containing 45 mg/mL protein. 122

Table 6.5. Recovery of Cat0059 after extraction process with ethyl acetate 122
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-DE</td>
<td>two-dimensional electrophoresis</td>
</tr>
<tr>
<td>a</td>
<td>lens radius,</td>
</tr>
<tr>
<td>[Ca^{2+}],</td>
<td>intracellular Ca^{2+} concentration</td>
</tr>
<tr>
<td>AAH</td>
<td>artificial aqueous humour</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variation (a statistical method)</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BODIPY-</td>
<td>4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid</td>
</tr>
<tr>
<td>Cx-</td>
<td>connexin</td>
</tr>
<tr>
<td>Da</td>
<td>daltons (atomic mass unit)</td>
</tr>
<tr>
<td>DF</td>
<td>dilution factor</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>IPG</td>
<td>immobilized pH gradient</td>
</tr>
<tr>
<td>Lp82</td>
<td>lens specific calpain protein (82 kDa)</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>M</td>
<td>molar (concentration)</td>
</tr>
<tr>
<td>M_w</td>
<td>molecular weight</td>
</tr>
<tr>
<td>OLCO model</td>
<td>ovine lens calcium-induced opacification model</td>
</tr>
<tr>
<td>P</td>
<td>probability</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>R^2</td>
<td>coefficient of determination</td>
</tr>
</tbody>
</table>
r  radial distance from the centre of the lens
rpm revolutions per minute
sd standard deviation
PAGE polyacrylamide gel electrophoresis
SJA6017 N-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal
SMCE store-mediated Ca^{2+} entry
TBS tris-buffered saline
TFA trifluoroacetic acid
TEMED tetramethylethylenediamine
Triton X-100 iso-octylphenoxypolyethoxyethanol (detergent)
TTBS tris-tween20 buffered saline
Tween20 polyoxyethylene (20)sorbitan monolaurate
Vhr volt hours
v/v volume per volume
w/v weight per volume
Dedication

To my beloved mother & To Hazel and Tricia,
who never doubted my capability in pursuing a PhD.
Chapter 1: Literature Review

1.1. Vision and Cataract

The lens of the eye is a vital component of the visual system. Its physiological function, in connection with the cornea, is to focus images on the retina by transmitting light through its transparent body. Its absolute clarity is essential as an optical component of the eye. When the lens loses its clarity and becomes opaque through the appearance of white or yellow cloudiness, it is called a cataract. Cataract initially affects distance vision and causes problems with glare and, consequently, a reduction in optical performance.

Fully opaque lenses lead to complete blindness, without any pain. Today, surgical replacement of the cataractous lenses with clear artificial ones is the only treatment for cataract. This is expensive and not readily available to everyone with a cataract. As a consequence, cataracts still account for the majority of cases of treatable blindness in the world. According to data from The Fred Hollows Foundation (2002), cataracts account for blindness in at least half of the estimated 45 million blind people worldwide.

Many internal and external factors have been identified as causing cataract. Among these are hereditary alterations in proteins, metabolic abnormalities, toxins, metal ion deposits, radiation, malnutrition, complications of other diseases (i.e. diabetes and glaucoma), physical injury and aging (Harding & Crabbe, 1984; Newell, 1992). In many cases, multiple factors are responsible for the development of lens opacity (cataractogenesis) and the biological mechanisms involved are complex. Consequently the exact aetiology of cataract formation is not fully understood (Hammond, 2001).

Extensive research is being pursued to understand the physiology of lens transparency and, therefore, to clarify the exact mechanisms by which cataracts form. The underlying mechanism that leads to cataractogenesis will provide the platform on which to develop a non-surgical therapeutic treatment to treat cataract.
1.2. Eye

The eye is an organ that detects light. The mammalian eye rests in a bony cavity (socket, or orbit) on the frontal surface of the skull. The structure of the mammalian eye is designed to focus light onto the retina. All of the individual components through which light travels within the eye before reaching the retina are therefore transparent.

The anterior outer covering of the eye is a transparent tissue, the cornea, which is continuous with the sclera that is visible as the white of the eye. A delicate membrane, the conjunctiva, covers the visible portion of the sclera. The cornea is richly supplied with sensory nerves that function to protect it and help maintain its transparency.

The cornea is continually kept wet and nourished by basal tears that lubricate the eye and help to keep it clear of dust. Tears are formed by tiny glands that surround the eye. The tear film is comprised of three layers: oil, water, and mucous. The lower mucous layer serves as an anchor for the tear film and helps it adhere to the eye. The middle layer is comprised of water. The upper oil layer seals the tear film and prevents evaporation.

Underneath the sclera is the second layer of tissue, the choroid, composed of a dense pigment and blood vessels that nourish the tissues. The choroid layer forms the ciliary body, which contains the smooth muscles. The ciliary body participates in changing the lens shape to focus light from different target distances to the innermost layer of tissue in the eyeball, the retina. The ciliary body in turn merges with the iris, a diaphragm that regulates the size of the pupil.

Between the cornea and iris (anterior chamber) and between the iris and lens (posterior chamber) are small spaces filled with aqueous humour (Newell, 1992). Aqueous humour has a refractive index of 1.335 and its density is slightly greater than that of water (reviewed by Davson, 1990). The large cavity behind the lens, iris, and ciliary body is filled with vitreous humour. Vitreous humour is a transparent jelly-like substance, consisting of 99% water. The remaining 1% is mostly collagen, cartilage matrix glycoprotein and hyaluronic acid (Balazes, 1994; Haddad, 1990). The microscopic fibres embedded within the gel of vitreous humour give rigidity to the spherical form of the eye, support the retina, and contribute to its attachment to the choroid.
1.3. Lens in Eye

The lens is a transparent, biconvex body of the eye that is encased in the lens capsule. It is a completely avascular tissue. The lens sits between the aqueous and vitreous humours and is held by suspensory ligaments from the ciliary body attached to the anterior and posterior lens capsule around its equatorial region (Figure 1.1).

The cellular structure is symmetric about an axis passing through its anterior and posterior poles, but asymmetric about a plane passing through its equator (Figure 1.2). The anterior epithelium of the lens is in contact with transparent nutritive fluid, the aqueous humour, and the posterior differentiating cells of the lens are in contact with the vitreous humour.

The lens is encased in a basement membrane, called the lens capsule, that is tightly attached to the epithelium. Type IV collagen and other extracellular matrix material are structural components of the lens capsule, and are synthesised by lens epithelium (Brinker et al., 1985; Bron et al., 2000).

Figure 1.1 Cross-section of a mammalian eye showing the position of the lens, which functions to focus incoming light on the retina (diagram modified from an Anatomical Chart, Skokie, Illinois, 1986).
Epithelium and fiber cells of the lens

The lens is composed of two types of cells; a single layer of epithelial cells on the anterior surface of the lens and a larger number of highly differentiated elongated fiber cells making up the bulk of its mass (Figure 1.2, A) (Manko, 2002). The lens epithelium is divided into central, pre-equatorial and equatorial zones. In general, cells of the central zone are mitotically quiescent whilst cells of the pre-equatorial are proliferative and produce new cells that migrate towards the equatorial zone where they terminally differentiate to form fiber cells (Francis et al., 1999; Paterson & Delamere, 1992).

![Figure 1.2](image)

**Figure 1.2** (A) Schematic drawing of cross section of the adult human lens (Gupta et al., 2005). The anterior surface of the lens is lined by a single layer of lens epithelial cells. Fiber cells constitute the central major mass of the lens and do not possess cell organelles or nuclei. The lens is enclosed in a lens capsule. (B) Arrangement of lens fiber cells and sutures in human lens (adapted from Brown, 2001).

The process of differentiation involves a large increase in cell volume due to cellular elongation, an increase in the concentration of the soluble structural proteins, crystallins, and the loss of cellular organelles and nuclei (Bassnett, 2002; Bassnett & Beebe, 1992; Manko, 2002). These are accompanied by the expression of an extensive range of fiber-specific proteins, cytoskeletal proteins like CP49 and filensin (Pitz & Moll, 2002), membrane associated proteins like MIP (Gorin et al., 1984) and MP20 (Grey et al., 2003; Louis et al., 1989), and the gap junction proteins, connexin46 and connexin50 (Lin et al., 1997; Paul et al., 1991).
The newly produced layers of fiber cells are laid over pre-existing fiber cells. This growth process continues throughout life, consequently, the lens consists of concentric layers with older fibre cells densely packed toward the centre of the lens (nucleus), and a gradient of young fibre cells, at different stages of differentiation, towards the outer layers (the cortex) (Hess et al., 1998) (Figure 1.2). The dominant structure at the poles is the suture lines, which are the junctions of fiber cells from opposite sides of the lens (Figure 1.2, B). The sutures maintain cellular diffusion and communication (Kuszak, 1995).

1.4. Components for the Transparent Lens

In order to focus the visual light on the retina, the lens must be crystal clear to transmit light without absorption or scattering, and have a relatively high index of refraction (Augusteyn and Stevens, 1998).

As the bulk of the lens mass is composed of elongated fiber cells, the regular arrangement of the lens fiber cells with minimal extracellular space provides a lens with small spatial fluctuations comparable to the wavelength of light (Bettelheim, 1985). Disorganisation of fiber cells, such as abnormalities of the fibre cell membranes and separation between fibre cells, leads to light scattering. The lens membrane is, however, only 5% of the total volume of the lens (Bettelheim, 1985). This means that the transparency of the lens is largely based on the transparency of the cytoplasm of fiber cells.

The highly differentiated fiber cell structure filled with the soluble proteins eliminates any fluctuation in refractive index between cells and creates a transparent lens environment. The spatial arrangement of lens cytoplasmic proteins (crystallins and cytoskeletal elements) and absence of light-scattering elements are vital factors contributing to lens transparency.

**Lens soluble structural proteins, crystallins**

The properties of the crystallins, a family of proteins, largely determine the transparency of the lens, and their high concentration in the lens fiber cells generates a high refractive index (Delaye & Tardieu, 1983; Horwitz et al., 1999). Crystallins are the major structural components of the lens comprising up to 70% of its dry mass in vertebrate lenses (Augusteyn...
There are three major classes of crystallins found in all mammalian lenses; α-, β- and γ-crystallins (Harding, 1991).

The predominant crystallins in mammalian lenses are the α-crystallins. The α-crystallins are distributed throughout the lens. They exist as large aggregates ($M_w$ around 800 kDa) of the subunits (polypeptides), $\alpha$A1, $\alpha$A2, $\alpha$B1 and $\alpha$B2 ($M_w$ ranging from 20 to 30 kDa) in all vertebrate species. These highly conserved proteins are members of a small heat-shock protein family (Ingolia & Craig, 1982) and function as molecular chaperones to prevent thermal aggregation of a number of enzymes and other lens structural proteins such as β- and γ-crystallins, by refolding partially denatured proteins (Derham & Harding, 2002; Horwitz, 1992). Hydrophobic surface interaction with α-crystallins may protect other proteins by kinetic competition between aggregation and interaction of unfolding proteins with α-crystallins (del Valle et al., 2002; Lindner et al., 2001).

Both β- and γ-crystallins are largely restricted to the lens fiber cells (Cvekl & Piatgorsky, 1996), although some β- and γ-crystallin expression has been recently reported in adult mammalian lens epithelial cells (Wang et al., 2004). β-crystallins are the second most abundant group of proteins in the lens, but their proportion and properties vary with development. The β-crystallins comprise a complex group of heteropolymers from different types of subunits, the acidic ($\beta$A1, $\beta$A2, $\beta$A3, and $\beta$A4, $M_w$ from 23 to 25 kDa) and the basic ($\beta$B1, $\beta$B2 and $\beta$B3, $M_w$ from 26 to 32 kDa) polypeptides.

γ-Crystallins are monomeric with molecular weights of 20 kDa, and are related to β-crystallins (reviewed by Augusteyn & Stevens, 1998). In most mammalian species, the production of γ-crystallins ceases at birth so high levels of γ-crystallins are observed in the centre of the lens, and they are absent in the cortex. The exception is rodent lenses where γ-crystallin synthesis persists after birth, but at a reduced rate (reviewed in Augusteyn and Stevens, 1998). γ-Crystallin distributions are the mirror image of the β-crystallins.

The normal folding of crystallins and their interactions are regulated in the peripheral region of the lens, where their rapid production occurs to provide the concentration necessary for the high refractive index of the lens (McAvoy, 1978; Piatigorsky, 1981). High concentrations of
the cytoplasmic proteins, largely crystallins, are packed into a glass-like short range spatial order, between crystallin and crystallin, as well as water and crystallin interfaces (Delaye & Tardieu, 1983; Zhao & Bettelheim, 1995). These close packing arrangements of crystallins within the cytoplasm are essential for the non-fluctuation in refractive index between crystallins, which forms the basis of lens transparency. Because of the lack of protein turnover in the lens, there are gradual increases in protein concentrations and, consequently, in the refractive index and hardness of the core area of the lens (Young & Fulhorst, 1966).

Post-translational modification of α- and β-crystallins by phosphorylation, truncation and deamidation, for example, occur during lens maturation and aging processes (David et al., 1994a; Miesbauer et al., 1994; Ueda et al., 2002). For instance, αA-crystallin becomes progressively phosphorylated with increasing age in the soluble fraction of mouse lens, with the phosphorylation form comprising more than one third of the total αA-crystallin by age 51 weeks (Ueda, 2002). Truncation of β-crystallins was observed during normal maturation in the rat lens (David et al., 1994a). Deamidation is a common post-translational modification, resulting in conversion of an asparagine residues to a mixture of isoaspartate and aspartate (Harding et al., 1984). Previous studies of proteins from the normal human lens have shown that deamidation of specific asparagine and glutamine residues occur during the aging process (Lampi et al., 1998; Lund et al., 1996; Takemoto & Boyle, 1998).

**Lens cytoskeletal elements**

In the cytoplasm of the cell, a complex network of fibrous proteins, the cytoskeleton, controls the overall organization of the cell. The cytoskeleton has an important role in stabilizing cell structure as the fiber cell differentiates. The cytoskeleton of mammalian cells consists of three different filament systems; microfilaments (diameter, 5-8 nm), intermediate filaments (10 nm) and microtubules (20-25 nm), with various associated proteins that crosslink these filament systems to each other and to other cell organelles (Bershadshky & Vasiliev, 1988; Pitz & Moll, 2002; Shoeman & Traub, 1990).

Microfilaments are the actin-containing filament system that helps maintain the cell structural framework, and play a role in the movement of cell components. Intermediate filaments contribute to cell stability, strength, cell structure, maintenance of cell-cell contacts, and nuclear structure (Pitz & Moll, 2002). Tubulin, the major protein of microtubules, provides
structural support and assists in intra-cellular locomotion and transport. All filament systems have a wide variety of associated proteins, e.g. microtubule-associated proteins (i.e. MAP1 and MAP2) and intermediate filament-associated proteins (i.e. spectrin and fodrin (brain spectrin)), that confer unique properties to the complexes and give rise to a variety of skeletal and dynamic structures (Shoeman & Traub, 1990).

In the ongoing process of lens development and differentiation, cytoskeletal elements are involved in coordinated proliferation and differentiation of lens epithelial cells into fiber cells. In differentiated fiber cells, cytoskeletal elements play a critical role in the stability of the lens cytoplasmic gel through their interaction with membrane proteins and crystallins. During the differentiating period, interactions of the prominent cytoskeletal proteins with the rapidly produced lens crystallins are necessary to establish the short-range order of crystallins in the lens cytoplasm and provide an environment favourable for a transparent cell structure (Matsushima et al., 1997). Clark (1990) suggested that weak non-covalent interactions are responsible for the transparent organization of cytoplasmic proteins. Consequently, the simplest modification in the proteins or their environment could influence the transparency or opacity of differentiating lens fibres.

Spectrin is the major protein component of the membrane skeleton and is composed of an \( \alpha/\beta \) heterodimer which forms a 200 nm extended tetramer filament (Bershadsky & Vasiliev, 1988). It cross-links actin microfilaments into an isotropic meshwork that is attached to the membrane. The spectrin-actin membrane skeleton underlies the plasma membranes of all cells and is important for cellular shape, membrane stability and deformability, as well as the formation of membrane subdomains (Morrow et al., 1997). Proteolysis of \( \alpha \)-spectrin (\( \alpha II \)-spectrin, non-erythroid \( \alpha \)-spectrin, or \( \alpha \)-fodrin) to discrete fragments is implicated in changes in cell shape and membrane morphology which occur in many cell types, including during terminal differentiation and aging of lens fiber cells (Lee et al., 2001).

Intermediate filaments undergo dramatic changes in subcellular distribution during the differentiation/maturation of the fiber cells. A type III intermediate filament protein, vimentin, is found in both the epithelial and differentiating fiber cells but is absent in mature fiber cells (Rammaekers et al., 1980). Vimentin is localised mainly in the cell cytoplasm and is highly conserved in a wide variety of species (Ellis et al., 1984).
Some cytoskeletal and filamentous structures may need to be eliminated as they are large enough to contribute to light scattering (Siew & Bettelheim, 1996). In the bovine lens, vimentin undergoes a dramatic reduction as indicated by immuno-histochemical staining, at a discrete stage during fiber differentiation, after the complete loss of the nucleus (Sandilands et al., 1995b). How the elements of the intermediate filament network are selectively removed is unknown, but the process may be influenced by post-translocational modifications and interactions with intermediate filament-associated proteins.

The beaded filaments are a lens-specific cytoskeletal structure composed of two fiber cell-specific intermediate filaments, named filensin (CP95 and CP115 in rat and bovine lens respectively) and phakinin (CP49) (Carter et al., 1995; Ireland & Maisel, 1984; Maisel & Perry, 1972). Filensin and CP49 are both proteolytically processed in the lens during fibre cell differentiation but, unlike the abrupt disappearance of vimentin, are retained in all fiber cells (Sandilands et al., 1995a). The control and regulation of these extensive proteolytic processes are important in models of cataractogenesis and lens function (Sandilands et al., 1995b).

Intermediate filament-associated proteins function as dynamic regulators of intermediate filament assembly (Hermann & Aebi, 2000). One important group of intermediate filament-associated proteins is the α-crystallins, where the solid-phase support of the beaded filaments facilitates the chaperone activity of the α-crystallin assemblies (Nicholl & Quinlan, 1994). Mutation in the CP49 gene in human (Conley et al., 2000; Jakobs et al., 2000) or knockout of CP49 in mouse (Sandilands et al., 2004) demonstrated a stabilising role of the beaded filaments in the lens, which is important for maintaining lens transparency.

Elimination of lens organelles
A striking feature of lens fiber cell differentiation is the abrupt degradation of intracellular structures that are large enough to scatter light, such as nuclei, mitochondria, Golgi bodies and the endoplasmic reticulum (Bassnett & Beebe, 1992; Piatigorsky, 1981). As a result, the terminally differentiated and aged fiber cells in the core of lens have no organelles, with very little active membrane transport and a low level of metabolic activity.
Mitochondria and endoplasmic reticulum disappear abruptly (Bassnett & Beebe, 1992), while nuclear breakdown is a relatively slow process, although functional inactivation is probably much more rapid. Caspases and nucleases have been suggested to play an important role in organelle degradation, which is different to apoptosis (Bassnett, 2002). Unlike apoptosis which would inevitably lead to a disruption in the uniform reflective index of the tissue, organelle degradation in the lens is tightly regulated and selective. It has been proposed that proteolysis of the gap junction proteins, connexins (Lin et al., 1997) and the cytoskeletal protein spectrin (Lee et al., 2001) accompany the sudden organelle breakdown.

1.5. Microcirculation

The continuous flow of aqueous humour supplies most of the metabolic needs for the lens through the epithelial cells and the network of gap junctions. The avascular lens is maintained mainly by the external circulation to the lens by the aqueous humour and the internal circulation system within the lens.

Aqueous humour is a transparent nutrient fluid formed by the ciliary body processes: diffusion, ultrafiltration, and active secretion. The aqueous humour enters the posterior chamber of the eye and flows through the pupil into the anterior chamber, and then leaves the eye via the Canal of Schlemm- a circular canal situated at the juncture of the sclera and cornea (Martini, 1992). The circulation of the aqueous humour in the anterior chamber is driven by a convective flow that results from the temperature difference between the iris and cornea. The rate of aqueous humour formation equals the outflow, so the intraocular pressure is maintained at a relatively constant level. The water and salts within the vitreous humour are in constant exchange with the aqueous. It has a very low flow rate compared to the aqueous humour (reviewed by Blazes, 1994).

The internal circulation system of the lens is thought to be generated by Na\(^+\), entering the lens extracellularly at all surface locations and flowing inward, crossing extracellular spaces at both anterior and posterior surfaces (Gao et al., 2004). The physiological role of the circulating current is to create circulatory system, or microcirculation, in the lens (Mathias et al., 1997). The flow of Na\(^+\) drives the flow of water in the same direction. The inward flow provides the cells of the lens with fluid, ions and metabolites.
The current around a lens is directed inwards at the anterior and posterior poles and outward at the equator (Robinson & Patterson, 1983). The outward flow directed toward the equator removes waste metabolic products and creates a well-stirred intracellular compartment.

The driving force for the inward flux is the electrochemical gradient between surface cell membranes and inner fiber cell membranes. The angular flow from equator to poles appears to be directed by the angular distribution of gap junctions in the outer shell of differentiating fiber cells (Mathias et al., 1991; Mathias et al., 1997).

Figure 1.3. (A) Internal ionic current flow around and through a normal lens (first suggested by Robinson and Patterson, 1983, diagram adapted from Mathias et al., 1997). Fluid movements were proposed to follow the same direction (Mathias et al., 1997). Angular current flows along the axis of fiber cells, rarely crossing a gap junction, while radial current flows across the fiber cells via gap junctions. (B) Lens Na⁺ transport. Na⁺ enters the lens along the extracellular spaces between cells and moves down its electrochemical gradient into fiber cells, where it returns to the surface via gap junctions. Three physiological zones; epithelium, peripheral shell of fiber cells and interior fiber cells are presented in italic letters (diagram adapted from Gao et al., 2004).

In the vertebrate lens, cells are coupled to their neighbours through the extensive network of gap junctions, which are clusters of transmembrane channels allowing small molecules (\(M_w \leq 1000\) Da), such as metabolites, ions and second messengers, to translocate from cell to cell (Bennett & Goodenough, 1978). The structural components of gap junctions are members of a family of membrane proteins known as connexins, which consist of four conserved transmembrane domains and two conserved extracellular loops. In the mammalian lens, three
connexins have been identified; Cx43 in epithelial cells (Kumar & Gilula, 1996) and Cx46 (Paul et al., 1991) and Cx50 (White et al., 1992) in differentiating fiber cells.

This intercellular pathway provides fibre cells with a low-resistance pathway for rapid diffusion to all cells, especially between the peripheral lens cells and the interior fiber cells (Donaldson & Kistler, 1993; Mathias et al., 1997). The differentiating fibre cells have their gap junction coupling conductance concentrated in the equatorial region. The distribution of gap junctions directs the intercellular current to the equatorial surface of epithelial cells. At the equatorial surface, highly concentrated \( \text{Na}^+ / \text{K}^+ \)-ATPase activity can transport the intracellular flux of \( \text{Na}^+ \) out of the lens, and complete the circulating current (Figure 1.3) (Gao et al., 2000; Tamiya et al., 2003).

The circulatory system can be regulated by modulation of the peripheral gap junctional conductance or membrane \( \text{Na}^+ \) conductance in the lens. The lens possess some receptors which could provide signal transduction for physiological regulation (Gupta et al., 1994; Ireland et al., 1994; Jacob et al., 1992). Systemic hormones and circulating neurotransmitters could therefore regulate the internal circulatory system and adjust the lens to the physiological state of the organism. As the lens is not innervated, adjustment would be slow relative to many physiological responses.

1.6. Physiological Zones in the Lens

Lens fiber cells function like a syncytium (a large region of cytoplasm that is not separated into individual cells, as in muscle fiber cells), as they appear to be electrically and dye coupled (Cohen, 1965). The degree and nature of the coupling in fiber cells, however, depend on location (Mathias et al., 1997). This suggests, in the lens, that there are three physiologically distinct zones: the anterior epithelium, the peripheral shell of fiber cells and the interior fiber cells.

The outer peripheral shell of fiber cells contains young differentiating fibre cells expressing a new complement of cytoplasmic and membrane proteins. Intracellular compartments, including organelles, still remain in this region. The degree of coupling between fiber cells is
not uniform, the highest cell-cell conductance is at the equator and the lowest towards the poles (refer to Figure 1.4.). Overall, gap junctional conductance is sensitive to intracellular pH on the outer peripheral shell, so the fiber cells in this region are uncoupled by a reduction in intracellular pH.

Then there is an abrupt transition at the scale of 0.7 radial distance (r) from the centre of the lens plotted over the lens radius (r/a, proportion of the lens radius). At this point, organelles and nuclei suddenly disappear at the final stages of lens cell differentiation (Bassnett & Beebe, 1992), and the gap junction channels abruptly lose their sensitivity to changes in pH to become uniformly coupled with the interior fiber cells (Baldo & Mathias, 1992).

![Figure 1.4](image)

**Figure 1.4 (A)** Lens morphology. Schematic diagram of lens morphology showing equatorial and axial sectioned planes and the dispersion of fiber cell nuclei. Directional axes indicated by white arrows aid in orientation in large-scale expression studies (a = lens radius, r = radial distance from the centre of the lens, c = circumferential, z = z-axial) (adapted from Jacob et al., 2004). r/a = 1 at the lens periphery and r/a = 0 at the lens centre. (B) Ideal construction of the degree of coupling between fiber cells by the intensity of shading where the lighter shade represents highest cell-to-cell coupling conductance (adapted from Mathias et al., 1997). Cells of the outer shell (r/a > 0.7) are rapidly and reversibly uncoupled in response to decreased pH, whereas interior fiber cells (r/a < 0.7) are insensitive to pH changes (Jacob et al., 2004; Mathias et al., 1997).

Cleavage of cytoplasmic tails of Cx50 by calpain is attributed to the loss of pH sensitivity at this transition region (Bassnett, 2002; Gong et al., 1998; Jacob et al., 2004). These modified connexin proteins keep channels open between the interior fiber cells (Figure 1.4) as the anaerobic metabolism of fiber cells generate a radial pH gradient in the lens with the acidity increasing toward the core of the fiber cells (Mathias et al., 1991).
The lens core is composed of anucleated fiber cells with no organelles and forms a functional syncytium (Shestopalov & Bassnett, 2000). It was proposed that neighbouring fiber cells are joined by a cytoplasmic bridge, providing a path by which large solutes (too large to permeate gap junctional channels) can slowly travel throughout the core of the lens (Kuszak et al., 1985; Mathias et al., 1997). This arrangement could facilitate the delivery of newly synthesized protein components to the aged and metabolically quiescent cells in the centre of the lens.

1.7. **Ca\(^{2+}\) Regulation**

Ca\(^{2+}\) is the most common intracellular signal transduction element in cells. The free cytosolic Ca\(^{2+}\) concentration, directly or indirectly, regulates many cellular functions, including cell proliferation, cell motility, contraction, secretion and gene transcription/expression (Berridge et al., 1998; Berridge et al., 2000; Clapham, 1995). Unlike many other secondary messenger molecules, Ca\(^{2+}\) can not be metabolised. Its prolonged high level within the cell is one of the important components in the cascade of events leading to pathological conditions and to cell death (Nicotera & Orrenius, 1998; Rizzuto et al., 2003). Therefore the cell tightly regulates intracellular Ca\(^{2+}\) levels through numerous binding and specialised extrusion proteins, so that any exogenous or internally generated Ca\(^{2+}\) load is rapidly controlled to maintain Ca\(^{2+}\) balance. Some cellular proteins have been adapted to bind Ca\(^{2+}\) tightly to lower or buffer free Ca\(^{3+}\) levels (i.e. calsequestrin) or to trigger secondary messenger pathways (i.e. calpain and calmodulin) (Baimbridge et al., 1992; Clapham, 1995; Heizmann & Hunziker, 1991).

In the human lens, a large fraction of Ca\(^{2+}\) was found to be in the bound or non-diffusible form (Duncan & van Heyningen, 1976), where free Ca\(^{2+}\) was approximately 1% of total Ca\(^{2+}\) (Duncan et al., 1989). A considerable amount of Ca\(^{2+}\) in the human and rat lenses was located in intercellular spaces, where it was bound to the outer leaflet of the bilayer (van Marle et al., 1997; Vrensen et al., 1995). The normal free intracellular Ca\(^{2+}\) level around 100 nM can be increased as much as 10-fold following cell-surface receptor stimulation for its cellular function, while extracellular Ca\(^{2+}\) levels can be found up to 2 mM (Berridge et al., 2000).

Ca\(^{2+}\) is involved in cell-signalling in lens epithelium and in the superficial fibers (reviewed in Duncan et al., 1994). Ca\(^{2+}\) enters the cells through a store-operated Ca\(^{2+}\) channel pathway by
a store-mediated Ca\textsuperscript{2+} entry (SMCE) mechanism, which is Ca\textsuperscript{2+} specific (Rosado & Sage, 2000). The intracellular Ca\textsuperscript{2+} concentration can be increased in lens epithelial cells by agonists (Williams et al., 1993) or mechanical stimulation (Churchill et al., 1996). In the epithelial cells of the rabbit lens, agonists that mobilize Ca\textsuperscript{2+}, whether by acting through G-protein or tyrosine kinase receptors, can modulate lens cell growth (Duncan et al., 1996). SMCE also mobilises Ca\textsuperscript{2+} from thapsigargin-sensitive intracellular stores into the cytoplasm.

The Ca\textsuperscript{2+} entry in SMCE is controlled by the filling state of the stores (Putney, 1990). For regulating Ca\textsuperscript{2+} levels during normal Ca\textsuperscript{2+} signalling, there is a continuous shuttling of Ca\textsuperscript{2+} between the endoplasmic reticulum (ER) and mitochondria in many cells. Those two organelles often serve as a Ca\textsuperscript{2+} buffering system for the cell. Normally the ER stores most of Ca\textsuperscript{2+} and there is very little in the mitochondria (Parekh & Putney, 2005). The high levels of Ca\textsuperscript{2+} in the ER are essential as a signal reservoir and also regulate synthesis and processing of proteins in the ER.

The intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}_i]) must be very tightly regulated in time, in space and in amplitude to be an effective cellular signalling mechanism. The consequence of the loss of intracellular Ca\textsuperscript{2+} homeostasis leads to various diseases, as demonstrated by the mutation or abnormalities of many of the proteins involved in Ca\textsuperscript{2+} regulation (Missiaen et al., 2000). Some mammalian proteins that are associated with [Ca\textsuperscript{2+}_i] are listed in Table 1.1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ca\textsuperscript{2+}-Binding Site</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tropomysin C</td>
<td>EF hand</td>
<td>Modulator of muscle contraction</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>EF hand</td>
<td>Ubiquitous modulator of protein kinases and other enzymes (MLCK, Ca\textsuperscript{2+} kinase II, adenylyl cyclase)</td>
</tr>
<tr>
<td>Calreticulin, retinin, visinin</td>
<td>EF hand</td>
<td>Activator of guanylyl cyclase</td>
</tr>
<tr>
<td>Calcinulin B</td>
<td>EF hand</td>
<td>Phosphatase</td>
</tr>
<tr>
<td>Calpain</td>
<td>EF hand</td>
<td>Protease</td>
</tr>
<tr>
<td>Insoluble phospholipid-specific PLCO</td>
<td>EF hand</td>
<td>Generator of InsPs and diacylglycerol</td>
</tr>
<tr>
<td>e-Azinin</td>
<td>EF hand</td>
<td>Actin-bundling protein</td>
</tr>
<tr>
<td>Annexin</td>
<td></td>
<td>Implicated in endo- and exocytosis, inhibition of PLC_i ion channel?</td>
</tr>
<tr>
<td>Phospholipase A2</td>
<td></td>
<td>Producer of arachidonic acid</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td></td>
<td>Ubiquitous protein kinase</td>
</tr>
<tr>
<td>G-actin</td>
<td></td>
<td>Actin-severing protein</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel</td>
<td></td>
<td>Effector of membrane hyperpolarization</td>
</tr>
<tr>
<td>InsP\textsubscript{4} Receptor</td>
<td></td>
<td>Effector of intracellular Ca\textsuperscript{2+} release</td>
</tr>
<tr>
<td>Ryothin receptor</td>
<td></td>
<td>Effector of intracellular Ca\textsuperscript{2+} release</td>
</tr>
<tr>
<td>Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger</td>
<td></td>
<td>Effector of the exchange of Ca\textsuperscript{2+} for Na\textsuperscript{+} across the plasma membrane</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}-ATPase</td>
<td></td>
<td>Pump of Ca\textsuperscript{2+} across membranes</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+} antiporters</td>
<td></td>
<td>Exchanger of Ca\textsuperscript{2+} for monovalent ions</td>
</tr>
<tr>
<td>BOPCAR</td>
<td></td>
<td>G protein-linked Ca\textsuperscript{2+}-sensing receptor</td>
</tr>
<tr>
<td>Cdkkemon</td>
<td></td>
<td>Fluctuator of muscle contraction</td>
</tr>
<tr>
<td>Villin</td>
<td></td>
<td>Actin organizing</td>
</tr>
<tr>
<td>Arrestin</td>
<td></td>
<td>Termination of photoreceptor response</td>
</tr>
<tr>
<td>S100\textsuperscript{B}</td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>EF hand</td>
<td>Ca\textsuperscript{2+} buffermodulator of nuclear hormone receptor</td>
</tr>
<tr>
<td>Calbindin</td>
<td>EF hand</td>
<td>Ca\textsuperscript{2+} buffer</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>EF hand</td>
<td>Ca\textsuperscript{2+} buffer</td>
</tr>
</tbody>
</table>
**Ionomycin**

Ionomycin (M<sub>w</sub> 747.07, C<sub>41</sub>H<sub>70</sub>CaO<sub>9</sub>) is an antibiotic that acts as a Ca<sup>2+</sup> ionophore. In crystal forms, ionomycin is joined in pairs by two hydrogen bonds to form a ‘dimeric’ globular structure having primarily lipophilic surfaces (Toeplitz et al., 1979). This allows the ionomycin to transport divalent cations across biological and artificial membranes by a carrier-type mechanism (Fasolato & Pozzan, 1989). The divalent cation selectivity order for ionomycin was determined by ion competition experiments to be: Ca > Mg >> Sr = Br (Liu & Hermann, 1978). Ionomycin is electroneutral, where Ca<sup>2+</sup> is exchanged for 2 H<sup>+</sup> resulting in no net charge movement (Dolber, 1981; Fasolato & Pozzan, 1989). Ionomycin binds Ca<sup>2+</sup> in the 7.0-9.5 pH range resulting in a complex exhibiting intensive UV absorption at 254 nm (Liu & Hermann, 1978). Ionomycin complexes and transports Ca<sup>2+</sup> in a one-to-one stoichiometry. Ionomycin is more effective than another Ca<sup>2+</sup> ionophore, A23187, as a mobile Ca<sup>2+</sup> carrier (Liu & Hermann, 1978).

Ca<sup>2+</sup> ionophores are valuable tools to manipulate intracellular ionic homeostasis. They allow movement of Ca<sup>2+</sup> between the major membrane-bound compartments of the cell, extracellular space, cytosol, endoplasmic reticulum, and mitochondria, by equilibrating the Ca<sup>2+</sup> gradient (Abramo & Duchen, 2003). It was first suggested that calcium ionophores directly translocate Ca<sup>2+</sup> across the plasma membrane (Pressman, 1975). The character of the increase in [Ca<sup>2+</sup>]<sub>i</sub> can be different depending on the type of cells and the ionophore concentration (Galitzine et al., 2005). Recent studies demonstrated that low concentrations (submicromolar concentrations) of ionomycin mobilizes Ca<sup>2+</sup> stores and subsequent Ca<sup>2+</sup> influx via SMCE pathway (Dedkova et al., 2000; Morgan & Jacob, 1994).

**1.8. Lens Ca<sup>2+</sup> Homeostasis**

Although animal cell membranes have a low permeability to Ca<sup>2+</sup>, they do have some small baseline Ca<sup>2+</sup> permeability. In the lens, the diffusible Ca<sup>2+</sup> is located in the intercellular spaces (Paterson & Delamere, 1982/1983; van Marle et al., 1997; Vrensen et al., 1995). Non-specific cation channel mechanisms, that are present in lens plasma membranes, are responsible for a slow leak of inward passive diffusion of Ca<sup>2+</sup> (Duncan et al., 1989).
The free Ca$^{2+}$ concentration of lens epithelium cells is maintained within the order of 100-200 nM by the cell transport processes of the plasma membrane Ca$^{2+}$-ATPase activity pump and plasma membrane Na$^+$/Ca$^{2+}$ exchangers (Duncan et al., 1993; Williams et al., 1993). Internal sequestration of Ca$^{2+}$ is also carried out by endoplasmic reticular Ca$^{2+}$-ATPase pump activity to shift Ca$^{2+}$ into the ER which functions as an intracellular Ca$^{2+}$ store (Churchill & Louis, 1999; Duncan et al., 1993).

A model of Ca$^{2+}$ homeostasis in the lens is proposed to explain how the interior fiber cells (mature fiber cells) maintain low [Ca$^{2+}$] in the absence of cell transporters to remove diffused Ca$^{2+}$ (Borchman et al., 1989a). The circulating current in the lens brings Ca$^{2+}$ into intercellular spaces and then back to the surface via gap junctions in the same manner as Na$^+$ (see Figure 1.3.). On the surface of the lens, the Na$^+$/Ca$^{2+}$ exchange and Ca$^{2+}$-ATPase activity transport the Ca$^{2+}$ out of the lens (Gao et al., 2004; Paterson & Delamere, 2004). For instance, the increased entry of Ca$^{2+}$ into clear lenses is offset by an increase in the activity of the Ca$^{2+}$-ATPase pumps (Borchman et al., 1989b).

Recent studies of Ca$^{2+}$ regulation have exhibited a sustained elevation in [Ca$^{2+}$], in differentiating fiber cells, with [Ca$^{2+}$], three-fold higher than in epithelial cells (Churchill & Louis, 2002). The studies showed that the persistent Ca$^{2+}$ elevation was due to an increased Ca$^{2+}$ influx. Cells with prolonged elevated resting [Ca$^{2+}$], are usually associated with disease states (Missiaen et al., 2000). Churchill and Louis (2002) proposed that the sustained elevation in the resting [Ca$^{2+}$], in the lens fiber cells may play a role in lens cell differentiation as a part of the normal development of the intact lens.

The circulatory system through the fiber cells, coupled with an active Ca$^{2+}$ regulatory mechanism by surface epithelial cells, is critical for maintaining Ca$^{2+}$ homeostasis of the lens and lens clarity. Disturbance in pathways connected to the regulation of Ca$^{2+}$ homeostasis, therefore, can lead to Ca$^{2+}$ pathological conditions of the lens, such as cataractogenesis.
1.9. \( \text{Ca}^{2+} \) Homeostasis and Cataract

Cataract is any opacity occurring in the lens, and is the universal pathological response to a variety of insults to the lens. It was first reported almost a century ago that there were marked changes in the normal ion levels of many human cataractous lenses. They have higher levels of lenticular \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) and lower \( \text{K}^+ \) content than normal lenses (Burge, 1909). Since then, a number of \textit{in vivo} and \textit{in vitro} studies have shown that the opacification is associated with cytosolic \( \text{Ca}^{2+} \) levels (Azuma \textit{et al.}, 1990; Duncan & Bushell, 1975; Hightower & Farnum, 1985; Marcantonio \textit{et al.}, 1986; Robertson \textit{et al.}, 2005; Sanderson \textit{et al.}, 2000; Truscott \textit{et al.}, 1990).

In many ageing tissues, increased \([\text{Ca}^{2+}]\) has been observed (Blalock \textit{et al.}, 1994; Ouanounou \textit{et al.}, 1999; Romero \textit{et al.}, 2002). With cataractogenesis, early studies showed that the elevation of total lens \( \text{Ca}^{2+} \) (Duncan & Bushell, 1975) was associated with the development of cortical opacification during ageing (Duncan & Jacob, 1984). Many cortical cataracts are highly localised in nature, leaving much of the lens intact and transparent (Duncan & Jacob, 1984; Gandolfi \textit{et al.}, 1990; Harding \textit{et al.}, 1982; Hightower \textit{et al.}, 1989). A degree of uncoupling between the fiber cells has been linked to the existence of clear and light-scattering areas within localised cortical opacities of human lens. Localised opacities show an increase in free \( \text{Ca}^{2+} \) content and no change of \( \text{Na}^+ \), which has been correlated with structural disruption (Duncan & Jacob, 1984; Harding \textit{et al.}, 1982; Hightower \textit{et al.}, 1989).

Duncan \textit{et al.} (1989) showed that the ageing human lens exhibited increased membrane electrical conductance and depolarisation of the membrane potential. The authors proposed that the activation of some non-selective cationic pathways occurred with ageing, which increased the permeability of the lens to ions. This process accounts for the catastrophic increase in lens \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) in human cortical cataractous lenses and is dependent on the membrane lipid composition (Borchman \textit{et al.}, 1989a; Delamere \textit{et al.}, 1991).

The lipid composition changes as lens age (Zeng \textit{et al.}, 1999) and with cataractogenesis (Borchman \textit{et al.}, 1993; Gooden \textit{et al.}, 1983; Paterson \textit{et al.}, 1997). Unlike lens \( \text{Na}^+ \), K-ATPases activities, which are independent of the lipid composition (Dean \textit{et al.}, 1996), the lens \( \text{Ca}^{2+} \)-ATPase, an important component of \( \text{Ca}^{2+} \) regulation, is dependent on the membrane
lipid composition (Borchman et al., 1989a; Delamere et al., 1991) and is sensitive to oxidative damage (Ahuja et al., 1999). It was reported that lens Ca\(^{2+}\)-ATPase activity was reduced by about 50% in human cortical cataracts (Paterson et al., 1997).

High [Ca\(^{2+}\)]\(_i\) is one factor (along with trans-junctional voltage and intracellular pH) which is known to acutely affect gap junctional conductance (Cooper et al., 1991; Crow et al., 1994). Disruption in the Ca\(^{2+}\) homeostasis affects internal networks essential for the microcirculation of the lens. Elevated Ca\(^{2+}\) uncouples lens epithelium cells, which are connected with numerous gap junctions by Cx43 (Cooper et al., 1991). In bovine lens epithelial cell studies, the rise of micromolar levels of [Ca\(^{2+}\)]\(_i\), induced by the presence of Ca\(^{2+}\) ionophores reduced the permeability of gap junctions (Crow et al., 1994). The rise of [Ca\(^{2+}\)]\(_i\) also increased the internal electrical resistance in the fiber cells, resulting in uncoupling of the fiber cells by a mechanism that is dependent on Ca\(^{2+}\) and calmodulin (Gandolfi et al., 1990).

Disruption of Ca\(^{2+}\) homeostasis in cells also compromises the regulation of Ca\(^{2+}\)-dependent protein activities (Table 1.1). Increased [Ca\(^{2+}\)]\(_i\) triggers the activity of Ca\(^{2+}\) dependent enzymes and induces changes in the protein profiles of various tissues. In the lens, where protein turnover is slow, the modification of proteins can lead to cataract.

Two important Ca\(^{2+}\)-dependent enzymes in the lens are the calpains (intracellular non-lysosomal cysteine proteases) (Murachi, 1983) and the transglutaminases (acyl-transferases) (Folk, 1980). They are both activated by the increase in [Ca\(^{2+}\)]\(_i\) in the lens (Lorand et al., 1998). They often compete for the same endogenous substrates, but one promotes the appearance of high molecular weight proteins by cross-linking (Clement et al., 1998; Folk, 1980; Lorand et al., 1991; Lorand et al., 1981; Shridas et al., 2001), whilst the other promotes the production of lower molecular weight forms by proteolytic degradation (David & Shearer, 1986, 1993; David et al., 1993; Shoeman & Traub, 1990; Yoshida et al., 1984).

1.10. The Calpain Proteolytic System

Calpains (EC 3.4.22.17, Clan CA) are found in nearly all animal tissues, with an absolute requirement for Ca\(^{2+}\) for activation (reviewed in Goll et al., 2003). There are at least 14
different calpain isoforms identified in mammals (reviewed in Huang & Wang, 2001). Some isoforms are ubiquitously expressed while others are tissue-specific.

In the lens, there are five calpains: ubiquitous calpain I, calpain II and calpain 10, and lens specific calpain Lp82 and Lp85 (reviewed in Biswas et al., 2004). Enzymatic, immunological and mRNA studies have shown that calpain II is the predominant isoform in the lens tissue of human (Shih et al., 2001), cow (Yoshida et al., 1985), rat (David & Shearer, 1986), mouse (Fukiage et al., 1997a), guinea pig (Fukiage et al., 1997a), rabbit (Fukiage et al., 1997a) and sheep (Robertson et al., 2005).

![Figure 1.5 X-ray crystal structure of calcium-free (inactive) human calpain II. A substrate binding cleft in the active site (circled) is located between two catalytic sub-domains of D-II (D-IIa and DIIb). Cys-105 (D-IIa), His-262 (D-IIb) and Asn-286(D-IIb) are the catalytic residues (adapted from Strobl et al, 2000).](image)

Lens calpain II from bovine and rat have a native molecular weight of approximately 110-120 kDa, and display characteristics similar to the calpain II isolated from other tissues (Murachi, 1983). Calpain II is a heterodimer with a variable large (80 kDa) catalytic subunit containing
an active site with four domains (Domain D-I, -II, -III and -IV) and a small (28 kDa) regulatory subunit with two domains (Domain D-V and D-VI) (reviewed in Goll et al., 2003).

The amino acid sequences of calpains from vertebrate species are highly conserved with over 90% homology among the mammalian calpains sequenced thus far (Goll et al., 2003). The heterodimeric structures for human calpain I and calpain II have recently been determined. There are close three-dimensional similarities between the two isomers, although they are encoded by different genes (Jia et al., 2001; Reverter et al., 2001).

Calpain activation

![Calpain activation](image)

**Figure 1.6** Calpain II activation *in vivo* in the presence of Ca$^{2+}$ and lipid bodies or membrane (adapted from Reverter et al., 2001).

In the absence of Ca$^{2+}$, structural constraints in calpain II hold two sub-domains IIa and IIb apart, thus maintaining the active site in a disassembled state (a, Figure 1.6). In the presence of Ca$^{2+}$, Ca$^{2+}$ binds to the EF hand motifs of domain IV and disrupts a salt bridge between Lys-7 (domain I) and Aps-154 (domain VI). This results in the liberation of the N-terminal of domain I and thereby initiates structural movement of IIa and IIb into close proximity. Consequently, Cys-105 (sub-domains IIa) can interact effectively with His-262 and Asn-286
(sub-domains IIb) to form a calpain active site, which facilitates or even triggers sub-domain fusion (b, Figure 1.6). This process could be assisted by Ca$^{2+}$ binding to negatively charged residues on both sides of the active-site cleft and lipid- and/or membrane-mediated Ca$^{2+}$ binding to a negatively charged loop in domain III. Also, Ca$^{2+}$ binding to domain IV is associated with lipid- and/or membrane-mediated activation of calpain II to promote other features of enzyme activation, including autolysis and subunit dissociation.

Table 1.2 Characterising functions of six domains of ubiquitous calpain I and calpain II (reviewed in Sorimachi, *et al.*, 1997).

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Domains</th>
<th>Key function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calpain I or</td>
<td>I</td>
<td>Proteolytic activity</td>
</tr>
<tr>
<td>Calpain II</td>
<td>II</td>
<td>Proteolytic activity</td>
</tr>
<tr>
<td>Large subunit</td>
<td>III</td>
<td>Ca$^{2+}$ binding, Lipid bodies/Membrane targeting</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>Containing EF hand motifs for Ca$^{2+}$ binding</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>Regulatory</td>
</tr>
<tr>
<td></td>
<td>VI</td>
<td>Containing EF hand motifs for Ca$^{2+}$ binding</td>
</tr>
</tbody>
</table>

*Limited proteolysis by calpain*

Cellular activity of calpain is closely regulated through Ca$^{2+}$-induced conformational changes in the calpains and by interaction (more detail discussed in section 1.12) with its specific endogenous inhibitor, calpastatin. The modulation of calpain activity by phospholipids and/or membranes and protein activators has also been reported (reviewed in Molinari & Carafoli, 1997).

Calpain I and calpain II have the same substrate specificity but differ in their affinity for Ca$^{2+}$. Calpain I and calpain II require approximately 3-50 μM and 400-800 μM Ca$^{2+}$ for half maximal activation respectively, based on data largely from bovine skeletal calpains (reviewed by Goll *et al.*, 2003). Both calpain I and II auto-proteolysize when incubated with Ca$^{2+}$ (Cong *et al.*, 1989; Dayton, 1982) without any affect on the specific activity of either enzyme (Edmunds *et al.*, 1991). Auto-proteolysis reduces the mass of the 80 kDa subunit of calpain II to 78 kDa, and the 28 kDa subunit to 20.5 kDa (Graham-Siegenthaler *et al.*, 1994). The auto-proteolysis (or autolysis) reduces the Ca$^{2+}$ concentration required for half maximal proteolytic activity of calpain II from 400-800 μM to 50-150 μM Ca$^{2+}$, and for calpain I from 3-50 μM to 0.5-2 μM Ca$^{2+}$. The physiological significance of autolysis remains controversial,
since both the autolysed and unautolysed calpains are capable of proteolytic activity. It has been reported that the peptides released during autolysis of subunits of calpain I have some chemotactic activities on neutrophiles (Kunimatsu et al., 1989) or immunocytes (Kunimatsu et al., 1995).

Lp82 is a splice variant of the muscle specific calpain, p94 (or calpain 3), but the structural changes make Lp82 more stable than p94 (Ma et al., 1998). Lp82 is expressed in foetal bovine and rat lenses (Ueda et al., 2001) but not in human lens. Lp82 levels rapidly decrease as the lens matures (Shearer et al., 1998). Lp82 enzymatic activity is the highest in the nucleus of the immature rat lens with a requirement of 30 μM Ca^{2+} for half maximal activity in vitro (Shearer et al., 1998).

Calpain-catalysed proteolysis proceeds in a limited manner, producing large polypeptide fragments rather than small peptides or amino acids (Goll et al., 1992). This limited cleavage by calpains results in alteration and modification of its substrate proteins, which leads to changes in their biochemical and morphological parameters. The calpains have a subsite specificity, preferentially cleaving peptide bonds having a leucine or valine residue (reviewed by Murachi et al., 1987). Other studies also indicate that the subsite specificity of the calpains is governed by conformation of the polypeptide chain (Croall et al., 1996; Stabach et al., 1997).

Another proposed role of calpains is the removal of damaged lens proteins, as proteins within the lens have a very slow rate of turnover (Taylor & Davies, 1987). The limited proteolysis may also facilitate further degradation of the substrate proteins, as the initial modification of the given protein by calpains can destabilize its structure and increase sensitivity to the attack from other cellular proteases. The complete degradation of proteins to amino acids is a concerted action between calpains, which convert proteins into polypeptide fragments, and the proteasome and lysosomal enzymes, which degrade polypeptides into amino acids (Goll et al., 1999).

The function of the calpains in the lens under normal physiological conditions is not well understood. Consequently, the physiological sites and targets of calpain action in cells and tissues can be important in understanding its functions. In the epithelium and cortical region
of the bovine lens, calpain II is the most dominant isoform of the calpains (Yoshida et al., 1985). A large portion of the substrates are proteins that are closely associated with membranes, including the cytoskeletal and other cytoplasmic proteins (Saido et al., 1994; Shoeman & Traub, 1990). Therefore it was suggested that proteolysis by calpains may play a role in eliminating filaments, organelles and cytoskeletal elements during lens development (David et al., 1994a; Marcantonio & Duncan, 1991; Marcantonio & Sanderson, 1997). In the nucleus of intact rat lenses, limited proteolysis of β-crystallins by calpain II was also observed during normal lens development (David et al., 1994a). The author suggested that this proteolysis may be required to increase the protein solubility and to increase both the refractive power and structural integrity of the lens.

Casein zymography for calpain activity
Detecting calpain activity in culture cells or tissue samples has been difficult due to the presence of various calpain isoforms and the presence of an excessive amount of the endogenous inhibitor calpastatin. Resolving the problem required chromatographic separation (Karlsson et al., 1985). More recently, a zymographic assay for calpains has been developed which separates calpains on casein-containing polyacrylamide gel under non-denaturing condition (Raser et al., 1995). Samples are loaded and electrophoresed into the gel under non-denaturing conditions, which ensures that different calpain isoforms in the sample are separated primarily according to their differing charge properties. This is in contrast to the size-dependent separation characteristic of SDS-PAGE. Calpain II runs into the middle of the non-denatured gel, whereas calpain I and Lp82 always stay within the top quarter of the gel, suggesting that calpain II has a greater negative charge.

The separated calpains are activated by incubating the gel in the Ca^{2+}-containing buffer upon the completion of the electrophoresis. Calpain activation causes the casein in the region to be digested into small fragments, which diffuse out of the gel. Calpain itself is also degraded by autolysis. Therefore staining the gel with protein-sensitive blue dye results in a blue gel with clear bands. The size and brightness of the cleared bands on the gel are dependent on the amount of calpain present in the loaded sample.

Casein zymography of calpain is also useful for studies to distinguish between reversible and irreversible inhibitors of calpain. When the irreversible inhibitor, E64, was incubated with calpain II, casein remained intact at the point of the calpain migration (Raser et al., 1995).
1.11. Cataract and Calpain

There have been several studies which have implied that the deregulation of calpains in the lens contributes to cataract formation. For example, uncontrolled calpain regulation was illustrated by increased calpain activities in the lens during aging without any change in their overall expression (Averna et al., 2001a; Benuck et al., 1996; Ibrahim et al., 1994; Kennessy et al., 1990; Manya et al., 2002; Saido et al., 1993b). Studies on human cortical cataractous lenses have revealed that the degree of the disruption in the lens ionic balance was correlated with the loss of protein and a loss of dry weight of cataractous lenses (Duncan & Bushell, 1975). In many instances, this was accompanied by the degradation of proteins which are substrates of calpains as shown in in vitro assays (Goll et al., 2003; Shoeman & Traub, 1990).

Calpain II was first shown to be present in lens cells by Yoshida et al. (1984). Subsequently, a number of experimental models have shown that there is a tight correlation between the products of calpain proteolytic degradation and the high Ca\(^{2+}\) content in organ cultured rat (Clark et al., 1999; Hightower et al., 1987; Sanderson et al., 1996; Truscott et al., 1990), bovine (Marcantonio & Duncan, 1991) and human lenses (Sanderson et al., 2000).

Calpain II-induced proteolysis and the precipitation of crystallins contribute to the underlying mechanism of cataract development in rodent lenses (David et al., 1993; David et al., 1994b; Kadoya et al., 1993), induced with selenite (David & Shearer, 1993), galactose (Azuma et al., 1990), diamide (Azuma & Shearer, 1992), Ca\(^{2+}\) ionophore A23187 and xylose (Shearer et al., 1991). These cataract models provide evidence for the calpain-associated degradation of lens proteins, including the major lens soluble structural proteins, \(\alpha\)- and \(\beta\)-crystallins, and some of the cytoskeletal and membrane proteins, that account for lens opacification (David & Shearer, 1986, 1993; David et al., 1993; Shoeman & Traub, 1990; Yoshida et al., 1984).

The proteolytic products of crystallins resulting from calpain-specific cleavage have been identified in both cataract models and mature lens animal models in extensive research by David and Shearer and their colleagues (David & Shearer, 1993; David et al., 1992; David et al., 1993; David et al., 1994a). The accelerated loss of the N-terminal extensions of \(\beta\)-crystallins by calpain II in selenite cataractous lenses of young rats resulted in opacification, compared to the slow continuous cleavage of \(\beta\)-crystallins in lenses of normal rats (David et
It was suggested that it was not the quantity of insoluble proteins, but the rate at which insolubilization occurs that actually determined the transparency or opacity. Truncation of the N-terminal extensions on β-crystallins made these crystallins less stable. Consequently, the truncated subunits precipitated and caused light scattering (Shearer et al., 1997). The removal of the N-termini caused a possible loss of hydrophilic regions and exposed the buried residues that are susceptible to oxidation (Lampi et al., 2001; Nakamura et al., 1999a) and deamination (Lampi et al., 2001).

The degraded products of α-crystallin produced by calpain II proteolysis in in vitro experiments were also found in in vivo selenite and sugar-induced cataract lens in studies conducted by Shearer and his colleagues (Azuma et al., 1995; Shearer et al., 1997). Truncation of the C-terminal of α-crystallins by calpain is known to cause the loss of the chaperone function of the α-crystallins (Horwitz, 2003). The truncated α-crystallins did not bind as well to the hydrophobic patch exposed on truncated β-crystallin, consequently, the α-crystallin fragments became entangled in the resulting insoluble pellets. Early studies have indicated that the chaperone activity of α-crystallin decreases upon in vitro treatment with calpain II, in senile and induced cataract in vivo, and in old human lenses (Cherian & Abraham, 1995; Kelley et al., 1993).

Major protein subunits of the cytoskeleton network are substrates for calpains, and as a consequence the cleaved cytoskeletal proteins have a reduced ability to cross-link (reviewed by Shoeman and Traub, 1990). In the lens, vimentin, spectrin and filensin, are cleaved rapidly by the calpains (Lee et al., 2001; Marcantonio, 1992; Truscott et al., 1990). In particular, the calpain-mediated cleavage of α-spectrin results in the rapid appearance of 150 kDa and 145 kDa degradation products (Fox et al., 1987; McGinnis et al., 1999; Wang, 2000). The calpain proteolysis of α-spectrin has been associated with a number of cataract models (Fukiage et al., 1998; Kilic & Trevithick, 1998).

Several studies have suggested that a substantial proportion of the calpains are associated with subcellular structures rather than existing as free calpains in the cell cytoplasm. In skeletal muscle, these structures are myofibrils (Kumamoto et al., 1992), whereas in other cells they may be cytoskeletal (actin) filaments (Lane et al., 1992). Ca^{2+} elevation in the diabetic and the actin depolymerization-induced-opacity model trigger calpain proteolysis of αII-spectrin.
and result in cortical opacification (Kilic & Trevithick, 1998). Human lenses, with age-related nuclear cataracts, display a higher density of finger-like membrane projections than transparent lenses of the same age (Bolyle & Takemoto, 1998).

Animal models for calpain-proteolysis in cataract
Selenite-induced cataractogenesis is a well-characterised cataract in vivo model. The rapid formation of a dense nuclear cataract is produced by a single subcutaneous injection of an overdose of sodium selenite to suckling rat pups aged 10-14 days (reviewed in Shearer et al., 1987). The oxidation of membrane sulfhydryl groups by selenite (a potent oxidizer of sulfhydryl groups) leads to impaired calcium homeostasis. The proposed mechanism is that calpain proteolysis causes changes in epithelium metabolism, Ca\(^{2+}\) accumulation and crystallin precipitation (Shearer et al., 1987; Shearer et al., 1992; Shearer et al., 1997).

Recently, connexin (Cx46) knockout (Gong et al., 1997; White et al., 1998) and (Cx46 for Cx50) knockin (White, 2002) mouse models have demonstrated there is a direct relationship between the rise of cytosolic Ca\(^{2+}\) levels and cataract formation. In these models, gap junction coupling conductance between mature fibres and the lens surface was modulated either up (Cx46 for Cx50 knockin mouse lenses) (Martinez-Wittinghan et al., 2003) or down (in Cx46 knockout mouse lens) (Gong et al., 1998). The absence of gap junction protein (Cx46 knockout) caused the loss of coupling of mature fiber cells. As a consequence, the efflux path for Ca\(^{2+}\) was blocked and Ca\(^{2+}\) accumulated in the mature fibre cells in a time-dependent manner, resulting in the development of a dense central opacity (Baruch et al., 2001; Gao et al., 2004). The mechanism of opacification was suggested to be over-activation of the lens specific calpain, Lp82, located in the central region of the mouse lens that produce cleaved crystallin, and protein aggregation for light scattering (Gong et al., 1997; Baruch et al., 2001).

The Shumiya cataract rat is a hereditary cataract model in which lens opacity appears spontaneously in the nuclear and perinuclear portions at 11–12 weeks of age (Shumiya & Nagase, 1988). Calpain-mediated proteolysis contributes to lens opacification during formation of this cataract (Inomata et al., 2001). Oral administration of aminoguanidine (an inhibitor of inducible nitric oxide synthase) to the Shumiya cataract rat suppressed the development of lens opacity and inhibited the calpain-mediated proteolysis of crystallins. The
author proposed that the aminoguanidine prevented cataract by suppressing Ca\(^{2+}\) influx into the lens cells.

The ovine cataract was the first reported occurrence of an inherited cataract in New Zealand Romney sheep (Brooks et al., 1982). The ovine cataract is an autosomal dominant trait in which partial cortical opacities can be detected within 1-2 months of birth (Brooks et al., 1982; Robertson et al., 2005). The pathology of the inherited cataract of sheep was investigated by Brooks et al. (1983), who showed that fibre cells underwent progressive swelling and lysis during cataract development. Cytoplasmic vacuolation of anterior epithelial cells was a notable feature. Progressive increases in water, Na\(^+\) and Ca\(^{2+}\) and decreases in K\(^+\) and Mg\(^{2+}\) were observed in ovine cataractous lenses. Cytosolic Ca\(^{2+}\) levels in mature ovine lenses were 10-fold higher than in normal lenses (Robertson et al., 2005). The presence of Ca\(^{2+}\)-dependent proteases were identified in the ovine lenses, and there was evidence that calpain-dependent proteolysis is important in this cataract (Robertson et al., 2005).

1.12. Calpain Inhibitors

*Endogenous calpain inhibitor and calpain regulation*

The most specific calpain inhibitor is the endogenous inhibitor protein, calpastatin. Calpastatin is a reversible inhibitor of calpain I and calpain II (Murachi, 1983). It is normally present in the cell in large excess compared with calpains. Calpastatin contains 4 repeated inhibitory domains of about 140 amino acids, and an N-terminal Domain L (110 amino acids) which lacks inhibitory activity (Croall & McGrody, 1994; De Tullio et al., 1998; Emory et al., 1987). The conserved residues within each of the four domains cluster into three regions called, A, B and C (Maki et al., 1991). Cells and tissues contain multiple forms of calpastatin, deriving from both post-transcriptional and post-translational modifications (Cong et al., 1998; Lee et al., 1992; Takano et al., 1999).

Ca\(^{2+}\) is required for calpain/calpastatin interaction and the Ca\(^{2+}\) requirement is thought to originate from the calpain molecules (Cottin et al., 1981; Otsuka & Goll, 1987). The most effective inhibition of the calpains by calpastatin requires that all calpastatin subdomains (A,
B and C) bind to the calpains at three sites on the calpain molecules; Domain IV, VI, and an area near the active site (domain II) (reviewed in Goll et al., 2003).

The *in vitro* Ca$^{2+}$ concentration required for calpastatin to bind to calpain is much higher than the 50-400 nM free Ca$^{2+}$ concentrations which exist in living cells (Becker et al., 1989; Berlin & Konishi, 1993). Hence the binding of calpastatin to calpain molecules may be poised to prevent any inappropriate or inadvertent calpain activity. The Ca$^{2+}$ concentration required for calpastatin to bind to calpain is, however, less than that required to initiate calpain proteolytic activity *in vitro* (Kapprell & Goll, 1989). Most immunolocalization studies found that calpain I and II and calpastatin are located exclusively intracellularly and that they are frequently co-localized in cells (Goll et al., 1992; Kumamoto et al., 1992).

One mechanism of retaining calpain activity in the presence of calpastatin is thought to involve re-locating calpain from a widely dispersed cellular distribution to a preferred location near the cell periphery. This occurs in response to a cellular signal, such as Ca$^{2+}$ influx (Goll et al., 2003). This mechanism would translocate the calpains away from the calpastatin whose location does not change (Gil-Parrado et al., 2003). Calpastatin remains in an aggregated form and normally localized close to the cell nucleus (De Tullio et al., 1999). Other studies have suggested that the cell localization of calpastatin may be correlated with the regulation and activation of calpain (Averna et al., 2001b). In the latter studies, it was shown that cAMP-dependent phosphorylation was responsible for the aggregation. Following an increase in intracellular Ca$^{2+}$, the dephosphorylation of calpastatin released it from its association and it became a soluble protein again. The authors proposed that this reversible phosphorylation process mediates the amount of calpastatin available for calpain inhibition.

*Exogenous inhibitors*

As a biochemical tool for understanding the detailed role of calpain-mediated cellular events and as a therapeutic agent for calpain-induced diseases, an ideal calpain inhibitor is required to have high selectivity towards calpain, possess good cell-permeability characteristics and low toxicity attributes. Most of the chemical structures being claimed as calpain inhibitors in the patent literature are peptide analogues. Calpain inhibitors have been designed from naturally occurring sources or by semi-synthetic and fully synthetic procedures.
One polypeptide inhibitor, mimicking the natural calpastatin, is a 27-residue peptide inhibitor derived from subdomain 1B of the repetitive domains of human calpastatin (Betts et al., 2003). This polypeptide calpain inhibitor displayed calpain specificity and was a potent inhibitor of calpains but its ability to penetrate the plasma membrane was poor. Recently, a new derivative (27-residue peptide with 11 poly-arginine peptides) has displayed effective penetration across the plasma membrane of neurons (Wu et al., 2003).

Almost all of the calpain inhibitors reported in the literature are small in molecular size and are designed to be active-site-targeted peptide analogues with calpain specificity. Early calpain inhibitors were the epoxysuccinyl peptides, including E64 (Azuma et al., 1991; Kadoya et al., 1993) and its analogues (Azuma et al., 1992). Irreversible covalent binding between the electrophilic centre of the inhibitors and the thiol group of the cysteine residue in the active site of calpain, blocked the proteolytic action of the calpain. E64 demonstrated some inhibitory effect to reduce cataract in in vitro and in vivo rodent models. There was, however, poor selectivity towards calpains and membrane permeability was low (Azuma et al., 1991; Kadoya et al., 1993). E64d, a structurally modified version of E64, with improved membrane permeability and increased calpain inhibitory activity, prevented induced cataract formation in the in vitro rat model (Azuma et al., 1992).

Peptide aldehyde calpain inhibitors function as competitive inhibitors of calpains, by binding calpains in a reversible Ca\(^{2+}\)-dependent way. Examples are leupeptin (Murachi, 1989), cBz-Val-Phe (MDL28170) (Mehdi et al., 1988), calpain inhibitor I, calpain inhibitor II, and calpeptin (cBz-Leu-nLeu) (Tsujinaka et al., 1988). The electric centre within these inhibitors reacts with the thiol group of the active-site cysteine of calpains. The aldehyde end group of the inhibitor binds the sulfhydryl group of the active site cysteine to trap the calpain. In leupeptin, the presence of the positively charged arginine residue reduces its cell permeability. cBz-Val-Phe (MDL28170) and calpeptin (cBz-Leu-nLeu) have improved membrane permeability and calpain selectivity over leupeptin and E64 groups, and exhibit better inhibitory effects on calpain-mediated cataractogenesis in in vitro rodent models (Sanderson et al., 1996). Unfortunately, they have limited selectivity for calpain over other proteases (Sorimachi et al., 1997). They are also pharmacologically unsuitable as anti-cataract agents due to their low water-solubility and toxicity towards lenses (Biswas et al., 2001; Sanderson et al., 1996).
Chapter 2: Experimental Rationale

2.1. Introduction

The unique structural and physiological properties of the lens are tightly integrated with the properties of lens proteins, in order to maintain its transparency. Any alteration or disturbance in this sophisticated system can affect the optical properties of the lens. In ageing and many ageing related diseases, there are post-translational modifications of proteins leading to unfolded or mis-folded proteins. Both older lenses and those with cataract have shown post-translational modifications of lens proteins (Harding, 2002).

Altered Ca\(^{2+}\) homeostasis (i.e. excessive increase in \([\text{Ca}^{2+}]_i\)) has frequently been linked to both age-related and non-age-related diseases, as Ca\(^{2+}\) is vital for regulating normal cellular functions. Among all other Ca\(^{2+}\)-dependent enzymes (Table 1.1), calpain has been implicated in a variety of important physiological processes, including signal transduction in cell proliferation and differentiation (reviewed by Ono et al., 1998). The consequence of uncontrolled calpain activation or a deregulated cellular calpain system, following the loss of cellular Ca\(^{2+}\) homeostasis, is the development of a number of pathological tissue conditions. In many ageing tissues with elevated intracellular Ca\(^{2+}\) levels, impairments to the proteolytic system leading to undesired protein cleavage or inefficient protein removal have been proposed to be a key aspect of cellular ageing (Nixon, 2003). Ageing is the most common factor in human cataract formation.

There is increasing evidence that experimentally induced and naturally occurring cataracts proceed along common pathways (Figure 2.1) (Harding, 2002). A rise in \([\text{Ca}^{2+}]_i\) has been reported in lenses obtained from a number of animal cataract models and from humans with cataracts. In such studies, the rise of intracellular Ca\(^{2+}\) level was correlated with proteolytic products from a limited cleavage of a number of proteins that have been linked with maintaining lens transparency.
2.2. Hypothesis for Current Research

The current research has been designed to investigate cataract formation associated with the loss of Ca$^{2+}$ homeostasis in the lens and to examine the involvement of calpain in cataractogenesis.

It is proposed that (i) Ca$^{2+}$ homeostasis is vital for lens transparency, (ii) the loss of Ca$^{2+}$ homeostasis leads to a malfunction in the regulation of calpain activity, and (iii) deregulation of calpain activity is one of the key mechanisms contributing to cataract formation.

Figure 2.1 A modified diagram of the changes seen in human cataract (adapted from Harding, 2002). This thesis investigates the role of Ca$^{2+}$ homeostasis and the involvement of calpain-activated proteolysis in the development of the cataract. Syneresis is exudation of the liquid component of a gel.
2.3. Research Methods: Ovine Lens Models

The use of ovine lenses in an *in vitro* system is a novel approach for the close examination of cataractogenesis. Unlike rodent lenses which are completely spherical and hardly accommodating, bovine and ovine lenses resemble the biconvex shape of human lenses (Augusteyn & Stevens, 1998). From the properties of the lens and the anatomy of its suspensory structure, it would be expected that bovine and ovine lenses are capable of accommodation. An intra-capsular accommodation mechanism is involved with active participation of metabolic processes. It is thought that lenses with accommodating power, like ovine and human lenses, share a metabolic pattern similar to muscle (ratio $\text{ATP/ADP} > 8$), whereas the non-accommodative lenses, like rodent, correspond to the metabolic pattern of the liver (ratio $\text{ATP/ADP} < 3$) (Wegener et al., 1995).

The larger size of ovine lens (average wet weight 1g for 3 - 9 months old sheep) compared with rodent lenses has the advantage of providing a valuable *in vitro* model for studying cataractogenesis. For example, in the lens culture system (*in vitro* model), a single intact ovine lens can be sustained and provide material for analysing biochemical and physical changes. There is also the advantage, at Lincoln University, of accessing intact ovine lenses from freshly killed animals, and delivering, with minimal time, the intact lenses to the *in vitro* culture system.

In addition, the *in vitro* cataractogenesis studies with ovine lenses at Lincoln University are cross-referenced to the naturally occurring ovine hereditary cataract flock maintained at Lincoln University (Brooks et al., 1982). The ovine cataract is bilateral (affecting two eyes equally) and progresses to full maturity within a year. The ovine hereditary cataract is a cortical cataract, which is a common type of human cataract (Duncan & Bushell, 1975; Robertson et al., 2005). It has a predictable cataract development, where the progression of the cataract formation in the lens can be monitored and examined by visual inspection with the same ophthalmologic equipment used for human patients. Assessment of whether calpain is associated with the development of cataracts in the ovine hereditary flock was investigated by applying calpain inhibitors to one eye of the sheep (Robertson et al., 2005). This *in vivo* ovine model has been a useful non-rodent model to examine the role of calpains in the mechanism of cataractogenesis (Robertson et al., 2005).
2.4. Outlines of Research Chapters

An ovine lens culture (in vitro) system and the inheritable ovine cataract (in vivo) model have been used to test the hypothesis of the involvement of Ca\(^{2+}\) and calpain in cataractogenesis. This involved visual measurements, biochemical and physiological analysis of changes occurring in Ca\(^{2+}\)-induced cataract, including analysis of lens proteolysis, calpain activity, microscopic changes and testing the efficacy of calpain inhibitors (including novel compounds) in the in vitro system. Novel calpain inhibitors were applied to the in vivo ovine model and their therapeutic potential assessed. There are four major experimental chapters (Chapter 3 to 6) in this thesis to test the hypothesis, followed by general conclusions with future research directions (Chapter 7). A brief description of each chapter follows:

Chapter 3 Viability of the intact ovine lens in culture system

An in vitro organ culture system was adapted as a major research tool for investigating the hypothesis, as the unique avascular nature of the lens makes the culture system an alternative controlled model for investigating the mechanism of cataractogenesis. The components of the culture media, as a replacement of the aqueous humour, are vital for sustaining intact lenses. It was imperative to assess the optimum requirements to sustain the ovine lens during the experiments, as the 'in vitro' ovine lens is a novel approach. Two culture media were assessed for measuring lens viability based on a LDH leakage assay. A culture system protocol was developed as a benchmark system for future research.

Chapter 4 Ionomycin induced opacity in ovine lens culture

The loss of Ca\(^{2+}\) homeostasis within the lens was induced by culturing intact ovine lenses with the Ca\(^{2+}\) ionophore, ionomycin. The characteristics of opacification were closely investigated and their linkage to proteolytic activity by calpain assessed to provide experimental evidence for the mechanism of cataractogenesis. Ca\(^{2+}\)-overloaded lenses in the culture system have produced a model to support the concept that certain lens proteins are essential for maintaining transparency and that Ca\(^{2+}\) homeostasis is important in maintaining the physiology of the normal lens.
Chapter 5  Application of calpain inhibitors to cataractogenesis models

In this chapter a calpain inhibitor, SJA6017, was used to confirm the involvement of calpain in Ca\(^{2+}\)-linked \textit{in vitro} cataractogenesis models. Two models were used and compared in relation to assessing the potency of calpain inhibitors in these cataract models. Novel calpain inhibitors were also tested for their efficacy in preventing opacification. Two novel inhibitors were then selected for application in the \textit{in vivo} inherited sheep model. For the therapeutic application of desirable anti-cataract drugs, young sheep with inherited cataract were topically administrated with eye-drops during their natural progression of cataracts. The progress of inherited cataracts was not prevented over the period of the trial. There was no evidence that the inhibitors penetrated into different parts of the eye.

Chapter 6  Drug profile of the novel calpain inhibitor, Cat0059

The data from Chapter 5 revealed that the inhibitory effects of novel anti-calpain drugs in the inherited ovine model were limited by their poor bioavailability in the lens. In this chapter, biological barriers in the eye were considered separately for testing the bioavailability of a novel drug administrated topically. The uptake of the drug by the lens \textit{in vitro} was investigated by measuring its permeability through the cornea and assessing the binding factors within aqueous humour and lens homogenates.

2.5. Outline of General Conclusion Chapter

Chapter 7  General conclusions and future directions

Highlights of experimental evidence in each experimental chapter are discussed in relation to the hypothesis. The research results contributed from the current studies have added to our existing knowledge (Figure 7.1). The application of calpain inhibitors, their contribution to the calpain hypothesis and the potential of calpain inhibitors as therapeutic agents in calpain-associated disorders are discussed. Overall conclusions are outlined along with suggestions for future research.
Chapter 3: Viability of the Intact Ovine Lens in Culture System

3.1. Introduction

The avascular lens has a specialised circulatory system to meet the metabolic needs of all its cells. The aqueous humour, which is in contact with the anterior region of the lens, provides nutrients and removes waste from the circulatory system within the lens. Due to this unique feature of lens physiology, an intact lens can be removed from the eye and sustained in a non-biological condition; an organ culture system which baths the lens in a synthetic media at its physiological temperature with the CO₂ level set in a sterilised chamber. Unlike a cell culture system which provides a group of viable cells to study, the intact lens in an organ culture system closely resembles its biological and physiological condition within the eye.

To date, \textit{in vitro} lens cultures have provided an alternative and controlled model to understand the mechanism of cataractogenesis, mostly with rodent (Sanderson \textit{et al.}, 1996; Truscott \textit{et al.}, 1990), bovine (Marcantonio & Duncan, 1991; Marcantonio \textit{et al.}, 1986) and human lenses (Hightower & Farnum, 1985; Sanderson \textit{et al.}, 2000). Many studies have shown that there are species differences, especially in aqueous humour dynamics and its composition (Aihara \textit{et al.}, 2003; Baeyens \textit{et al.}, 1997; Berman, 1991; Mermoud \textit{et al.}, 1996). Despite the success with animal lens culture systems, there is little information on the dynamics of the ovine lens aqueous humour and its linkage to the lens. Since the influence of aqueous humour dynamics on the lens \textit{in vitro} conditions is vital, it is important to assess the viability of ovine lenses in culture systems.

Furthermore, it is vital to establish whether a culture medium can replace the aqueous humour and sustain viable intact lens within a culture system. Eagle's minimum essential medium (EMEM or MEM) is one of the most widely used synthetic cell culture media (Eagle, 1976). It is complex but a defined system without serum or growth factors. A simpler medium, artificial aqueous humour (AAH), was formulated originally based on the analysis of human aqueous humour (Davies \textit{et al.}, 1984).
In addition to selecting the type of medium, there are other factors which may influence the viability of the lens in the culture system. For example, minimizing the time between death of the donor and delivery of the lens to the culture system is important for successful culture. Sanderson et al. (1996) found that in human lenses the time had to be less than 48 h if the lenses were to remain viable over a 2 week culture period in EMEM (Sanderson et al., 1996). In addition, isolated lens must be maintained under a sterilised condition in order to prevent or limit growth of fungus. Removal of wastes from the lens, by changing the culture media regularly, is also important.

Cell viability is assessed by monitoring signs of cell death, which are normally evaluated by the quantification of plasma membrane damage. Lactate dehydrogenase (LDH) is a stable cellular cytoplasmic enzyme but it is rapidly released into cell culture media if the plasma membrane is damaged. Consequently, the amount of LDH activity detected in the culture medium correlates to the proportion of lysed cells (Decker & Lohmann-Mattens, 1988; Martin & Clynes, 1991; Masanet et al., 1988; Szekeres et al., 1981).

The ovine lens is a novel experimental animal model in cataractogenesis studies. Assessment of the viability of the ovine lens in an organ-culture system is an important step to determine whether the culture system can be adopted to study the physiological status of the lens in a cataractogenic-induced aqueous environment. In the current chapter, the cell viability of the ovine lens was examined by culturing intact ovine lenses in two different culture media, EMEM or AAH. From the results, a culture system protocol was developed as a standard for future research.
3.2. Methods

Preparation of culture medium
The EMEM culture medium for normal culture (EMEM, pH 7.4) is a bright-light red solution (due to the presence of Phenol Red-Na pH indicator as a component), prepared from Minimum essential medium powder (or MEM, purchased from Sigma, product number M0643, 2003), 26 mM NaHCO₃, 0.02 mg/mL gentamycin (antibiotic, from Sigma) and 2.5 μg/mL amphotericin B (anti-fungal, from Sigma) in dH₂O. Pre-treatment EMEM solution (pre-EMEM, pH 7.4) was prepared with Minimum essential medium powder, 26 mM NaHCO₃ and 1 x Antibiotic Antimycotic Solution (100 units/mL penicillin G, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B, from Sigma) in dH₂O. Artificial aqueous humour (AAH, pH 7.3) culture media is a colourless solution that was adapted from the formulation described by David et al (1984): 130 mM NaCl; 5 mM KCl; 1 mM CaCl₂; 0.5 mM MgCl₂; 2.5 mM D-glucose; 20.0 mM HEPES; 5.0 mM NaHCO₃. All culture media were sterilised immediately using 0.2 μm pore size filters (VacuCap® 60 Filter Unit: 0.2 μm pore size Supor® Membrane (hydrophilic polyethersulfone)) into autoclaved bottles.

Lens sample collection
Groups of ovine eye globes from 9-12 months old lambs, were collected from a local slaughterhouse immediately after slaughter and delivered to the laboratory for lens dissection. Pairs of lenses from the eyes of each animal were kept together. Lenses were dissected within 2 h of death of the animal using the posterior approach. The intact lenses were immediately placed in a sterile culture dish containing 10 mL of corresponding culture media per lens (see experimental protocols). The entire lens was submerged with its anterior epithelium facing upward in the medium. They were then incubated at 37 °C, 5% CO₂ in a sterilised chamber.

Experimental protocols for lens viability with two culture media.
Immediately after dissection, two groups of intact lenses were placed either in EMEM (n = 6) or AAH (n = 4) for measurement of spontaneous LDH release. At the same time, another two groups of intact lenses were placed either in EMEM containing 2% Triton X-100 (n = 5) or AAH containing 2% Triton X-100 (n = 4) for determining maximum LDH release. All lenses were incubated at 37°C, 5% CO₂ in a sterilised chamber for the total 25 h experimental period. A series of media samples (100 μL per lens) were collected during the experimental period. Triplicates of each culture medium sample were analysed in the cytotoxicity assay.
Experimental protocol for lens viability with pre-EMEM treatment.
Immediately after dissection, each of twelve pairs of intact lenses was placed in 10 mL pre-EMEM media. After 30 min incubation period, they were transferred to a culture dish containing 10 mL EMEM per lens, and were incubated at 37°C with 5% CO₂ under sterile conditions for the total 48 h experimental period. All media were completely changed at the end of the first 24 h period. For the LDH leakage assay, 5 mL of EMEM medium per lens were collected at the end of each 24 h incubation. Triplicates of each culture medium sample were analysed in the cytotoxicity assay.

Visual monitoring of cultured lenses
Lenses were photographed using a digital camera (Sony Cybershot DSC-F505V) fitted to a stand (Haiser R 3 XA). A transparent flat bottom culture dish containing a lens and medium was placed on black grid lines (1 x 1 mm) with a white background light (Radiographic Supplies, Christchurch, New Zealand). Images of the anterior epithelium of the lens were taken with an image resolution of 1856 x 1392 pixels as a RGB true colour JPEG image.

Lens opacity grade scoring using an image analysis system
The software used to grade the opacification captured was Image Pro-Plus v4.1. A macro script was created to automatically analyse the digital images of lenses (pixel size 1856 x 1392) placed on a 1 mm x 1 mm black grid. The grading system was developed on the basis of selecting predefined pixel RGB values, after area defining and sharpening of the image.
Part 1: The programmed system predicts total blobs (which are clear squares formed by the 1 mm x 1 mm black grid) from the total lens area. Due to the consistent measurements of the camera position and settings, the measurement of pixels/cm is a constant. This allows the macro to analyse multiple user-specified images automatically. Part 2: The system sharpens the image using a sharpening filter built into the software. The macro identifies the actual blobs visible through the opaque parts of the lens by selecting predefined pixel RGB values.
Part 3: From the constant pixel/cm ratio, the macro is able to compare the calculated actual blobs against the total predicted blobs calculated from the size of the lens. This value is shown as a percentage and used as the score of the lens. Full opaque lens are graded as 100, while clear/transparent lens are graded as 1.

Lens viability assay- cytotoxicity detection
Cell viability was measured by LDH levels in the culture media, using a cytotoxicity detection assay kit (Roche Applied Science, Cat. No.1 644 793). Evaluation of cell
cytotoxicity required information on the LDH activity level in the assay media (AAH or EMEM (background control)), LDH activity level released from the lenses in normal medium (spontaneous LDH release), and the amount of LDH released from lenses in EMEM or AAH with 2% (v/v) of non-ionic detergent, Triton X-100 (iso-octylphenoxypolyethoxyethanol) for inducing maximum LDH leakage (maximum LDH release). Triton X-100 is one of most commonly used non-ionic detergents for solubilizing membrane proteins during isolation of membrane-protein complexes (Barbero et al., 1983; Schubert et al., 1983).

Culture medium (100 μL/well) was mixed with 100 μL LDH kit reaction mixture in an optically clear flat bottom 96 well microplate, then incubated at RT for 30 min in the dark. The final concentration of Triton X-100 in the 200 μL assay volume was 1% (Triton X-100 does not interfere with reagents at this concentration). For quantitative measurement of LDH levels in the samples, 1000 U/mL of LDH (lactic dehydrogenase from bovine heart, Sigma) was diluted with assay medium (AAH or EMEM) from 0 (background control) to 10 mU/100 μL to generate a standard curve. All samples were assayed in triplicate. The absorbance of the samples at 492 nm was measured and the background at 660 nm subtracted.

Relative cell LDH leakage levels of the lens in normal culture medium (spontaneous LDH) over maximum releasable LDH activity level in culture containing 2% Triton X-100 (maximum LDH) were calculated for assessing cytotoxicity in the culture medium.

Relative percentage of normal lens cytotoxicity was determined according to the formula:

% cytotoxicity = [1-(spontaneous LDH - maximum LDH) / maximum LDH] x 100.
3.3. Results

Ovine lenses in the opacification scoring system

An ovine lens is biconvex with an asymmetric structure about a plane passing through its equator (Chapter 1, Figure 1.1 and 1.2). A lens from a 9-12 months old sheep has between 10-13 mm diameter of surface anterior region (complete circle), with a wet weight range between 700 and 1000 mg. Black suspensory ligaments remained attached around the equatorial regions of dissected lenses and indicated the outer boundary of lenses fully suspended in the culture media.

The grid can be seen as clear prominent black lines through a transparent lens (Figure 3.1, A), or as blurry lines as the lens becomes opaque and scatters light (Figure 3.1, B and C). According to the visual image analysis developed at our laboratory, a score below 11 represents a clear/transparent lens in the EMEM (Figure 3.1, A), while a score above 90 indicates a densely opaque lens (Figure 3.1, C). A score of 50 represents partial opacity (Figure 3.1, B), resulting in a blurry area with semi-dense opacification. This data showed direct correlation of the visual assessment of opacification with the digitally analysed score of opacification.

![Figure 3.1](image-url)

**Figure 3.1** Digital images of three ovine lenses in the culture media (EMEM) on a grid including their opacification scores (or relative transparency score system) based on digital visual analysis. (A) clear/transparent lens with the score of 8.5, while (B) partially opaque lens with 50 and (C) fully opaque lens with the score of 93, both induced by treatment with Ca$^{2+}$ ionophore, ionomycin, over the total 24 h experimental period.
**Lens transparency**

Apparent black grid lines indicated that lenses cultured in EMEM (n = 5) maintained their transparency unchanged after 26 h incubation period with average opacification score of 11.4 ± 2.9 (Figure 3.2, A and B). Lenses cultured in AAH at 5 h were slightly more opaque (18.4 ± 2.0, Figure 3.2, a) than lenses incubated in EMEM but overall they were relatively transparent. These lenses, however, became significantly blurry around the equatorial region at 26 h (Figure 3.2, b).

Lenses cultured with 2% Triton X-100 for 26 h were swollen and developed a strong eggy odour with approximately 35% and 45% opacification in AAH and EMEM media respectively (Figure 3.2, C and c). The colour of the EMEM medium changed from light red to yellow, indicating a fall in pH of the EMEM as a result of acid production from lysed cells induced by 2% Triton X-100. All lenses treated with 2% Triton X-100 for inducing maximum LDH release displayed the formation of blurry opacification within the inner fiber cells (white arrows in Figure 3.2). A distinctive barrier between the outer-shell of the cortex fiber cells and the inner shell of nuclear fiber cells is obvious in these lenses.

![Figure 3.2 Digital images of transparency of ovine lenses and their opacification scores (± sd, 1 = clear/transparent lens, 100 = full opacity), after culture in EMEM (A, B, n = 5), AAH (a, b, n = 4), EMEM containing 2% TritonX-100 (C, n = 5) and AAH containing 2% Triton X-100 (c, n = 4) for 5 h (T5) and 26 h (T26) experimental period. All lenses were cultured in an incubator at 37°C, 5% CO2. White arrows in C and c are pointing to the internally generated barrier in 2% Triton X-100 treated lenses.](image-url)
Cytotoxicity assay

The spontaneous LDH leakage from lenses cultured in normal media was compared with the maximum releasable LDH leakage of lenses by culturing them in the media containing 2% Triton X-100 (Figure 3.3). Lenses cultured in media with 2% Triton X-100 showed a rapid increase of LDH levels and within a 2-3 h incubation period reached their maximum releasable LDH activity. In comparison, the LDH level from lenses cultured in AAH and EMEM were low during the initial 6 h incubation period.

![Graph](A) AAH (B) EMEM

**Figure 3.3** A plot of mean LDH leakage levels (mU/100 μL medium) released from ovine lenses during culturing in AAH (A) or EMEM (B). Spontaneous LDH activity (n = 5) in normal culture medium (Δ) was compared to maximum releasable LDH activity (n = 4) where culture medium contains 2% Triton X-100 (■) (EMEM+Triton or AAH+Triton).

The relative cytotoxicity of a culture medium was based on the LDH leakage levels from lenses during a 24 h culturing period. Relative cytotoxicity level of lenses cultured in AAH gradually increased over the incubation period. At the end of 24 h, the LDH levels released from lenses cultured in AAH were approximately 50% of their maximum releasable LDH activity level (Figure 3.4, A). On the other hand, the relative cytotoxicity levels released from lenses cultured in EMEM were unchanged and remained approximately 20% of the cytotoxicity over a 24 h incubation period (Figure 3.4, B).
Figure 3.4 A plot of relative cytotoxicity (%) of normal ovine lens during culture in AAH (A) and in EMEM (B) for 24 h. % Cytotoxicity = \[ 1 - (\text{spontaneous LDH} - \text{maximum LDH}) / \text{maximum LDH} \] x 100.

**Pre-EMEM treatment**

At the end of the first 24 h incubation period followed by a 30 min of pre-EMEM treatment, the LDH activity released from lenses \((n = 12)\) was \(149 \pm 16 \text{ mU/100 \muL}\). At the end of the second 24 h period, the LDH activity of the same lenses were \(35 \pm 9 \text{ mU/100 \muL}\). This is 23% LDH leakage compared to the one measured at the end of the first 24 h culture period. The apparent reduction of LDH activity in the second 24 h incubation period indicates that the lenses were in steady condition in the culture system over this period, and that the pre-treatment did not affect lens viability.
3.4. Discussion

Current data have demonstrated the importance of providing the metabolic conditions for sustaining intact young ovine lenses in a culture system. Cytotoxicity data in our studies have revealed that the culture medium, EMEM, can sustain ovine lenses for a 24 h incubation period. The viability of the lenses coincided with maintenance of the transparency of the lens over the incubation period. The main ingredient of the culture media (EMEM) is a minimum essential medium (MEM) proposed by Eagle (1976). In addition to basic salts components, MEM contains high concentrations of amino acids and metabolites to meet the special nutrient requirements for facilitating cell growth in monolayers (Eagle, 1976). When the viability of human lens in the EMEM was tested by the capacity of intact lens to synthesize proteins, the data showed that intact human lens transferred from the eye into the culture system had remarkable resilience in the EMEM culture medium (Sanderson et al., 2000). Therefore, the EMEM without serum or any additional supplements provides an ideal culture environment to maintain the viability and transparent status of intact lens.

On the other hand, the current data revealed that ovine lenses cultured in artificial aqueous humour (AAH) medium developed approximately 50% cytotoxicity by the end of a 24 h incubation period. This increased cytotoxicity level coincided with a significant development of lens opacification during the same incubation period. The formulation of AAH is composed of basic salts with a glucose and buffer component (HEPES) based on analysis of human aqueous humour (Davies et al., 1984). However, the AAH media contains only glucose to satisfy the basic metabolic needs of the avascular nature of the lens. Aqueous humour contains various nutrients along with ionic components (reviewed in Berman, 1991). For instance, in most mammalian species, the concentration of amino acids in the aqueous humour is higher than in plasma, due to the active transport across the ciliary epithelium to meet the metabolic needs of avascular tissues including the lens and cornea (Berman, 1991).

In the eye, aqueous humour is continuously replaced in exchange for a constant supply of metabolites and for eliminating wastes from surrounding ocular tissue including the lens and cornea (Freddo, 2001). In the culture system, the lenses were placed in 10 -13 fold volume media (10 mL per lens) which is in excess of the natural volume of aqueous humour (approximately 500 to 800 μL in 3 - 6 months old ovine eye). Despite the buffering system
containing HEPES to minimise changes from pH 7.4, the lens were only viable for a few hours. These data show that specific nutrient components, at optimum levels, are needed to maintain the viability of ovine lens and their transparency over long-term incubation periods. There are two types of intercellular communication, ionic and metabolic (Duncan & Wormstone, 2001). It has been shown in several systems, including the lens, that metabolic communications can be severed whilst ionic communications remain intact (reviewed in Duncan et al., 1998). The AAH culture medium was demonstrated to be effective in maintaining a constant level of ions in rat lens for up to 24 h by other research (Davies et al., 1984; Lucas et al., 1986).

There are also possible species differences in the extent of communication between different regions of the lens, and the pattern of communication can change as the lens develops (Duncan & Wormstone, 2001; Mathias et al., 1997). AAH has been adapted to sustain rodent and human lenses by Cornish et al. (2002) and Sanderson et al. (1999). In young ovine lenses, the metabolic needs for a rapidly developing tissue may be a key factor influencing their viability and could possibly explain the importance of adding supplements with metabolic characteristics to the culture medium.

To minimise the infection of the organ culture system, antiseptic treatment techniques have been applied to the freshly dissected lens as an initial step to maintain the viability of the lens (Sanderson et al., 1996; Sanderson et al., 2000). Current data showed that the antibiotic/antimycotic solution treatment prior to transfer to the culture system had a minor effect on lens viability. This is thought to be an ‘initial response’ of the lens taken out of its biological system to a synthetic environment. The LDH leakage level, however, was dramatically reduced in the second 24 h period, which indicates the lens subsequently stabilises in the in vitro system. Changing the culture medium (EMEM) daily confirmed that the lenses needed at least 24-48 h to adjust to new aqueous conditions after removal from the eye (Sanderson et al., 1996).

Another interesting observation was that two regions in the lens were identified which were different in their internal opacity when the lens was treated with the 2% non-ionic detergent, Triton X-100. The proximity and location of the boundary between the two regions closely reflect the boundary between the two physiological zones in the lens. One zone is the
differentiating outer-shell fiber cells and the other, the interior mature (differentiated) fiber cells as described in Chapter 1 (Figure 1.4). At this boundary, between the zones, there is an abrupt change in the composition of fiber cell membrane proteins and their membrane associated proteins. The connexins, which are important components of the gap junctions in the interior mature fiber cells, become less pH sensitive, and more uniform in their conductance within this area (reviewed in Mathias et al., 1997). Triton X-100 is commonly used for solubilizing membrane proteins. Consequently it promotes cell death and the cells leak the maximum releasable LDH from the lens into the culture medium. These data showed that cell death promoted by addition of 2% Triton X-100 treatment resulted in the identification of two physiological zones in the lens, a peripheral shell of fiber cells and the interior fiber cells.

Taken together, a protocol for ovine lens culture system has been developed in this thesis as a standard protocol for future research using cultured ovine lens. The culture system protocol has been developed after assessing the viability of lens with different types of culture medium.

The developed protocol for a culture system is:

1. **Within 2 h of the death of the animal, the lenses are dissected using the posterior approach and incubated in a pre-EMEM solution (MEM, pH 7.4; 26 mM NaHCO₃; 0.25 µg/mL amphotericin B; 100 units/mL penicillin G; 100 µg/mL streptomycin) for 30 min in a laminar flow hood.**

2. **Thereafter, each lens is submerged in 10 mL EMEM (MEM, pH 7.4; 26 mM NaHCO₃, 0.02 mg/mL gentamycin; 2.5 µg/mL amphotericin B) and incubated in a sterile chamber (37°C with 5% CO₂) for a settling period of at least 2 days, prior to the application of any experimental treatment.**

3. **Daily replacement of the culture media is recommended.**
Chapter 4: Ionomycin Induced Opacity in Ovine Lens Culture

4.1. Introduction

70% of human cataracts are cortical cataracts and are associated with changes in the internal ionic content of the lens (Duncan & Bushell, 1975). Culture studies with lenses from rats (Clark et al., 1999; Sanderson et al., 1996; Truscott et al., 1990), cattle (Marcantonio & Duncan, 1991) and humans (Sanderson et al., 2000) have provided evidence for an association between increased level of cytosolic Ca\(^{2+}\) and increased amounts of proteolytic products with changes in the optical properties of the lens. These cataract models have implied that over-activation of calpain proteolysis is a key mechanism contributing to Ca\(^{2+}\) associated cataractogenesis.

The lens culture system has been subjected to various cataractogenic agents to initiate Ca\(^{2+}\)-induced opacification. For example, intracellular Ca\(^{2+}\) levels of lenses have been raised by various means such as: a hyper-Ca\(^{2+}\) environment (Biswas et al., 2004; Hightower & Farnum, 1985), osmotic stress by ouabain (Strophanthidin, a Na\(^+\)/Ca\(^{2+}\) exchange inhibitor) (Marcantonio et al., 1986), mechanical stimulation (Churchill et al., 1996), a Ca\(^{2+}\)-influx stimulator (a sulfhydryl reagent p-chloromercuriophenyl sulfonate) (Truscott et al., 1990) and Ca\(^{2+}\) ionophores (A23187 and ionomycin) (Marcantonio & Duncan, 1991; Sanderson et al., 1996; Sanderson et al., 2000).

Among cataractogenic agents that induce a rise of cytosolic Ca\(^{2+}\) levels, the Ca\(^{2+}\) ionophores (A23187 and ionomycin) have been reported as selective and potent Ca\(^{2+}\) transporters (Liu & Hermann, 1978; Toeplitz et al., 1979). Unlike other cataractogenic agents which stimulate Ca\(^{2+}\) influx indirectly, Ca\(^{2+}\) ionophores elevate cytosolic Ca\(^{2+}\) level directly by transporting Ca\(^{2+}\) across biological membranes and/or by store-operated Ca\(^{2+}\) entry pathways. In a cell-line culture system with various tissues, Ca\(^{2+}\) ionophores have been numerously used for manipulating cytosolic Ca\(^{2+}\) concentrations to study Ca\(^{2+}\) signalling systems. However, there have been only limited applications of Ca\(^{2+}\) ionophores in lens culture systems reported so far.
In comparison to cell cultures comprising single layers of cells, the effect of Ca$^{2+}$ ionophores-initiated Ca$^{2+}$ influx into cells of the lens is largely dependent on the physiological and structural properties of the lens. The physiological properties of cells in different regions of the lens is tightly linked to the unique micro-circulatory mechanism coordinated by the cells of the lens. As a consequence, the disturbance of Ca$^{2+}$ homeostasis could be varied within the lens and, consequently, so be the characteristics of the lens opacification.

In this chapter, we have characterised a Ca$^{2+}$-induced cataractogenesis, using ionomycin and organ-cultured ovine lenses. It is named the ovine lens Ca$^{2+}$ ionophore-induced opacification or OLCO model. The OLCO model has provided us with an important experimental modelling system to investigate the correlation between intracellular Ca$^{2+}$ levels and lens proteolysis, leading to cataractogenesis. In the present studies, the characteristics of opacification were closely investigated to understand the effect of a sustained high Ca$^{2+}$ concentration on the ovine lens system and to search for a mechanism of cataractogenesis. This system provides an opportunity to understand the importance of Ca$^{2+}$ homeostasis in relation to lens transparency and to test the hypothesis that over-activation of calpain-induced proteolysis is one of the key mechanism of Ca$^{2+}$ associated cataractogenesis.
4.2. Experiment 1: Ionomycin induced opacification in intact ovine lens

The objective of this experiment is to characterise the opacification associated with increased intracellular \( \text{Ca}^{2+} \) levels using a \( \text{Ca}^{2+} \) ionophore (ionomycin) and a \( \text{Ca}^{2+} \) chelator (EGTA) in the presence of the \( \text{Ca}^{2+} \) ionophore.

The involvement of calpains in developing opacification was assessed by determining if there was any \( \text{Ca}^{2+} \)-induced proteolysis of lens proteins and the degree of calpain activity.

4.2.1. Methods

Preparation of treatment solutions
Normal culture medium, EMEM, (MEM, pH 7.4; 26 mM \( \text{NaHCO}_3 \), 0.02 mg/mL gentamycin; 2.5 \( \mu \text{g/mL} \) amphotericin B, refer to Chapter 3) prepared and sterilised. This EMEM contains 1.8 mM \( \text{Ca}^{2+} \). An extra 1.8 mM \( \text{Ca}^{2+} \) was added to the EMEM to make a total of 3.6 mM \( \text{Ca}^{2+} \) in the medium, labelled as a EMEM-3.6. 1 mg/mL concentration of ionomycin (C41H70CaO9, Sigma) was prepared by solubilising ionomycin in DMSO. For \( \text{Ca}^{2+} \) chelated medium solution, 5mM EGTA was solubilised in the EMEM-3.6 medium, so that free \( \text{Ca}^{2+} \) concentration in the medium is largely restricted (less than 1 \( \mu \text{M} \)) (Sanderson et al., 2000). All treatment solutions were prepared under sterile conditions.

Lens culture
A group of sheep of the same breed (9 – 11 months old) were slaughtered at a local abattoir and their eye globes were immediately collected. The lenses were dissected within 2 h of the death of the animal using the posterior approach, and cultured in EMEM according to the culture system protocol described in Chapter 3.

Experimental protocol
After a settling period of 2 days, a number of intact lenses of similar sizes were randomly divided into 3 groups for the three experimental treatment groups. Each group of lenses was
transferred and cultured for a 48 h experimental period in one of three treatment solutions; EMEM-3.6 (Control); EMEM-3.6 + 5 μM ionomycin (Iono); EMEM-3.6 + 5 μM ionomycin + 5 mM EGTA (Iono+EGTA). Lenses with Iono+EGTA treatment were cultured in the solution of EMEM-3.6 with 5 mM EGTA for 30 min, first, to chelate external Ca$^{2+}$. Then an adequate volume of ionophore solution (1mg/mL DMSO) was introduced to the Ca$^{2+}$-chelated lenses to make 5 μM ionomycin concentration in the medium.

The treatment solution of all the lenses was replaced with fresh solution after the first 24 h of incubation. At the end of 48 h treatment period, all the lenses were removed from the medium, rinsed with dH$_2$O and carefully rolled on filter paper to remove any adhering non-lens tissues and vitreous humour. The wet weights of the clean lenses were measured and lenses were stored at -80°C. Lens transparency was monitored before and at 48 h after the application of the treatment.

**Colorimetric calcium assay**
A frozen whole lens was placed in 3 volumes of ice cold Buffer-A (20 mM Tris-HCl, pH 7.5; 2 mM DTT). This was homogenised using a Polytron PT 3100 homogeniser (Kinematica AG, Littau, Switzerland) with a Polytron PT-DA 3012/2T mm aggregate tip for 20 s at 25,000 rpm speed. 200 μL of the homogenate was immediately collected for colorimetric Ca$^{2+}$ assay, using Sigma Arsenazo III Kits (from Sigma). The Ca$^{2+}$ concentration of all the samples was determined at 600 nm using a spectrometer (UNICAM, UV4-100, England).

**Analysis of lens proteins**
Immediately after the collection of aliquots of the homogenates for the calcium assay, an equal volume of ice cold Buffer-B (20 mM Tris-HCl, pH 7.5; 2 mM EDTA; 2 mM EGTA; 2 mM DTT) was added and mixed. These homogenates were centrifuged in a Beckman J2-MI centrifuge for 30 min at 48, 500 x g (4°C). The supernatant and pellet (particulate and insoluble material) were collected. The supernatant was labelled ‘soluble protein’. The pellet was re-suspended in dH$_2$O and centrifuged in order to wash off any remaining soluble protein residues. Then the pellet was re-suspended again in 2 mL of 6 M urea for the extraction of proteins that were bound to the insoluble lens material, and centrifuged in a Beckman J2-MI centrifuge for 30 min at 48, 500 x g (4°C). The supernatant was collected and labelled ‘urea soluble protein’. The concentrations of total soluble and urea soluble samples were obtained using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, U.S.A.).
Casein gel zymography

Calpain activity in the lenses was determined by zymography (Raser et al., 1995). A two-phase casein zymogram mini-gel (a casein gel topped by a stacking gel) was prepared. A ten percent non-denaturing polyacrylamide mix (0.225 M Tris-HCl, pH 7.5; 10% acrylamide (37.5:1); 0.06% ammonium persulfate; 0.06% TEMED) was co-polymerised with pH 7.5, 0.05% casein (Hammersten grade, BDH) in a BioRad Mini-PROTEAN® 3 Casting Frame at 0.75 mm thickness. Once the casein gel set, a 4 percent non-denaturing stacking gel for sample loading was prepared (0.125 M Tris-HCl, pH 6.8; acrylamide (37.5:1); 0.1% ammonium persulfate; 0.1% TEMED). Once the stacking gel set, the gel was pre-run at 4°C in zymography running buffer (25 mM-HCl, pH 8.3; 192 mM Glycine; 1 mM EGTA; 1 mM DTT) in a Mini-Protean III (BioRad) electrophoresis tank at 125 V for 15 min. A known amount of soluble protein sample was mixed with zymogram loading buffer (0.04 M Tris-HCl, pH 6.8; 6.5% glycerol; 0.005% bromophenol blue) and was loaded on to the pre-run casein gel. All gels were run with the zymogram running buffer at 125 V for 150 min at 4°C. After electrophoresis, the gel was removed from the tank and incubated with gentle shaking overnight at room temperature in calcium incubation buffer (20 mM Tris-HCl, pH 7.4; 20 mM Ca²⁺; 10 mM DTT). Gels were rinsed with distilled water for 15 min then stained with Simply Blue™ Safe Stain (Invitrogen) and destained several times in dH₂O. Transparent bands appeared on a blue stained background gel.

SDS-PAGE and Immunoblotting (Western detection)
The urea soluble proteins were separated on an SDS-PAGE gel to assess changes in protein profiles. Known amounts of urea soluble protein samples from various treatment groups were mixed with SDS sample loading buffer (0.08 M Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.02% bromophenol blue) and denatured at 95°C for 4 min. After cooling, the samples and molecular markers (Precision Plus Protein™ Standards (All Blue) or Precision Plus Protein™ Unstained Standard, from BioRad) were loaded onto a NuPAGE® 4-12% Bis-Tris Gel, 1.0 mm x 10 well (gradient pre-cast gel, Invitrogen) in an X-cell SureLock™ Mini-Cell (Invitrogen) electrophoresis tank. NuPAGE® MES SDS Running Buffer (50 mM Tris-HCl, pH 7.3; 50 mM MES; 0.1% SDS; 1 mM EDTA) was added to the tank to run electrophoresis at 200 V for 45 min at RT. After the completion of the electrophoresis, the gel was removed from the gel cassette and rinsed with distilled water several times. The gel was then stained

52
with the GelCode® Blue Stain Reagent (PIERCE) for 1 h and incubated and destained several times in dH₂O.

For immunoblotting (western detection), after electrophoresis, the gel was removed and rinsed with transfer buffer (25 mM Tris-HCl, pH 8.3; 192 mM Glycine). The gel and PVDF membrane (Milipore Corporation, Bedford, MA, USA) were assembled together, and proteins from the gel were electroblotted onto the PVDF over 1 h at 100 V in cold (4°C) transfer buffer. Once the separated proteins on the gel were transferred onto the PVDF, the membrane was incubated with 5% blocking buffer (5% non-fat dry milk in TTBS (20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.05% Tween 20) for 45 min. Then the membrane was rinsed several times with TTBS and probed with monoclonal antibody to vimentin (clone V9; Santa Cruz) at 1:1000 in 3% blocking buffer by incubating at room temperature for 1 h. The membrane was then washed several times with TTBS and incubated with a mixture of secondary antibodies (goat-anti mouse, 1:2000 and Precision StreptTactin-AP conjugate, 1:5000 (from BioRad, for labelling unstained molecular marker)) in 3% blocking buffer for 1 h at RT. The gel was washed with TTBS and followed by TBS for several times to remove the Tween20. The detection of protein was visualised with AP Substrate Kit premixed colour developing solution (BioRad).

Two-dimensional electrophoresis (2-DE) analysis
Proteolysis of lens proteins was assessed by 2-DE. Spots from 2-DE gels were identified by comparison with previously mapped lens proteins (Robertson, Thesis 2003). 75 μg soluble protein sample from a treatment group was re-hydrated in 185 μL of re-hydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 2% IPG buffer pH 3-10 (Bio-Rad), 2% glycerol, 0.001% bromophenol blue). The first dimension was focussed on an immobilized pH gradient (IPG) gel strip (11 cm, pH 3-10, ReadyStrip™ IPG Strip, BioRad, USA) in a BioRad PROTEAN® IEF Cell for 6.5 h at 5000 V (33000Vhr) at 20°C. The IPG strip containing lens proteins was incubated twice for 15 min in 10 mL equilibration buffer (6 M urea, 50 mM Tris, 30% glycerol, 2% SDS, 0.001% bromophenol blue; pH 8.8). During the first incubation, the equilibration buffer contained 2% DTT, and during the second incubation the equilibration buffer contained 2.5% iodoacetamide. The IPG strip was fixed to a 12% Bis-Tris gel (Criterion™XT Precast Gel) with running buffer containing 1% agarose (pH 8.6) for molecular weight separation of proteins from the IPG strip in a BioRad Criterion electrophoresis setup. The second dimension gel was run using a power supply (Bio-Rad PowerPac 200, Hercules, CA, U.S.A.) for 60 min at 200 V with XP-MES Running Buffer
(Bio-Rad). The spots on each gel were stained with Simply Blue™ Safe Stain (Invitrogen), and scanned at a resolution of 100 pixels per inch using an Expression 1600 flat bed scanner with a transparent lid (Epson, Long Beach, U.S.A.).

4.3.2. Results

*Lens opacification induced by ionomycin*

Ovine lenses were cultured for 2 days in culture media alone (Control) or in the presence of 5 μM ionomycin (Iono) or in the presence of 5 μM ionomycin with 5 mM EGTA (Iono+EGTA) (Figure 4.2). All lenses in the control group remained transparent during the 48 h experimental period in a Ca$^{2+}$ concentration (3.6 mM Ca$^{2+}$) that was twice the normal concentration in the standard culture medium (Control, Figure 4.2).

Exposure to ionomycin resulted in the complete loss of lens transparency (Iono, Figure 4.2). The opaque lenses appeared noticeably swollen, and the original firmness of the intact lens was largely reduced. A white opaqueness mixed with a slightly pink pigment appeared in the opaque lenses indicating fluid uptake by the lens from the surrounding culture medium that contains Phenol Red·Na. The involvement of calcium in ionomycin-induced lens opacity was investigated by adding the Ca$^{2+}$ chelator, EGTA, to the medium containing ionomycin and Ca$^{2+}$ (Iono+EGTA, Figure 4.2). The lens opacity was largely inhibited by Ca$^{2+}$ chelation.

There was only restricted light scattering around the equatorial region and in the centre of the anterior surface region. These lenses (Iono+EGTA) showed no sign of lens hydration and their mean wet weight was not different to normal lenses (Table 4.2).

Figure 4.2 Digital images of ovine lens after culture for 48 h in EMEM-3.6 (Control), EMEM-3.6 + 5 μM ionomycin (Iono) and EMEM-3.6 + 5 μM ionomycin + 5 mM EGTA (Iono+EGTA). Note: EMEM-3.6 media contains a total of 3.6 mM Ca$^{2+}$, whilst normal EMEM medium contains 1.8 mM Ca$^{2+}$.
**Lens wet weights**

Lenses were weighed at the end of the 48 h experimental period (Table 3.1). The mean wet weight of lenses cultured with ionomycin was 30% higher than the control lenses ($P < 0.001$) (Table 4.2). The mean weight of lenses cultured in the Ca²⁺ chelated medium, containing the same concentration of ionomycin (Iono+EGTA), was not significantly different from the mean weight of lenses cultured in the normal media (Control). The dry weights of the lenses showed no significant changes between the treatments (Table 4.1).

**Table 4.1** Mean wet weight ($n = 6$), mean dry weight ($n = 3$) and mean Ca²⁺ concentration ($n = 6$) of ovine lenses ($±$ sd) after culture for 48 h in EMEM-3.6 (Control), EMEM-3.6 + 5 μM ionomycin (Iono) and EMEM-3.6 + 5 μM ionomycin + 5 mM EGTA (Iono+EGTA). EMEM-3.6 media contains total of 3.6 mM Ca²⁺.

<table>
<thead>
<tr>
<th>Lens treatment</th>
<th>Average weight (g)</th>
<th>Dry weight (g)</th>
<th>Ca²⁺ level (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.774 ($±$ 0.071)</td>
<td>0.313 ($±$ 0.017)</td>
<td>0.88 ($±$ 0.01)</td>
</tr>
<tr>
<td>Iono</td>
<td>*0.942 ($±$ 0.043)</td>
<td>0.296 ($±$ 0.015)</td>
<td>*3.17 ($±$ 0.02)</td>
</tr>
<tr>
<td>Iono+EGTA</td>
<td>0.793 ($±$ 0.023)</td>
<td>0.321 ($±$ 0.006)</td>
<td>*1.19 ($±$ 0.01)</td>
</tr>
</tbody>
</table>

Two-sample T test, *$P < 0.001$ relative to both control and Iono treatment group.

Total Ca²⁺ level

The total Ca²⁺ level of lenses from various treatment groups was measured at the end of the experiment analysed by colorimetric assay (Table 4.1). The correlation of the involvement of Ca²⁺ with lens opacity in the OLCO model was investigated by comparing the changes in transparency of lenses treated with ionomycin in Ca²⁺ chelated medium (Iono+EGTA) to lens treated with ionomycin alone (Iono). Analysis of Ca²⁺ levels showed that the mean total Ca²⁺ concentration in ionomycin-induced opaque lenses was three-fold higher than control lenses in EMEM, while Ca²⁺-chelated lenses were only slightly higher than control lenses.

Calcium-dependent protease activity

The increase in Ca²⁺ caused by ionomycin treatment could activate several enzymes including the calpains. Zymography was used to determine the amount and types of calpain in the lens following each type of treatment. Transparent bands represent Ca²⁺-activated proteases.
As a control, soluble proteins from lung and lenses were separated on casein gels and incubated in a buffer containing no calcium (20 mM Tris-HCl, 10 mM DTT, 1 mM EGTA). No bands developed in the absence of Ca\(^{2+}\) (Gel-2, Figure 4.3) indicating that the bands detected were all Ca\(^{2+}\)-activated proteases.

![Figure 4.3 Casein zymography of calpains extracted from ovine lung and normal ovine lenses cultured for 72 h in EMEM. Gel-1 (+Ca\(^{2+}\)) on the left side was incubated with Ca\(^{2+}\) buffer (20 mM Ca\(^{2+}\)), while Gel-2 (-Ca\(^{2+}\)) on the right side was incubated with 1 mM EGTA containing no Ca\(^{2+}\). Each well was loaded with 100 μg of soluble protein and run at the same time.]

As calpain activation is followed by autolysis, the decreased levels of calpain indicate that there was activity during the period preceding analysis. Four distinct bands appeared on the casein gel assay from lenses cultured in normal media (Control, Figure 4.4). These bands represent three calpain isoforms and an autolyzed form: Lp82, calpain I, calpain II and autolyzed calpain II, which were previously identified by using western blots with antibodies for each specific calpain isoform (Robertson et al., 2005). According to the intensity of each band (Figure 4.4, Control), calpain II, in both intact and autolyzed forms, was the dominant isoform in the ovine lens. The high level of autolyzed calpain II in these lenses indicated the level of protease activity present in the normal cultured lenses. On the other hand, Lp82 and calpain I were relatively minor components of the calpain isoforms.

Lp82 was present in both lenses cultured with ionomycin and with calcium-chelated ionomycin, as well as in normal lenses. The intensity of each Lp82 band was similar between the groups, suggesting there is no involvement of Lp82 in the ionomycin-induced opacification. On the other hand, calpain I activity was absent in lenses cultured with ionomycin, suggesting activation of calpain I occurred (Figure 4.4). Lenses cultured with the Ca\(^{2+}\) chelator and ionomycin contained similar calpain I activity levels as the normal lenses.
Figure 4.4 Casein Zymography of calpains present in 100 µg of soluble protein from lenses after culture for 48 h in n EMEM-3.6 (Control), EMEM-3.6 + 5 µM ionomycin (Iono) and EMEM -3.6 + 5 µM ionomycin + 5 mM EGTA (Iono+EGTA). EMEM-3.6 media contains total of 3.6 mM Ca\(^{2+}\). An extract of lung was used as control (Lung). Duplicate zymogram run for each experimental group.

Calpain II activity levels in the 3 different experimental groups were closely examined by loading lower amounts of soluble proteins (25 µg) on the casein zymogram from the same samples (Figure 4.5). Enzymatic activity by intact calpain II in normal lenses was similar to that in EGTA-ionomycin treated lenses. The intact calpain II activity in ionomycin treated lenses was, however, distinctively lower than the other two groups. With 25 µg of soluble protein loaded to the gel, no difference was seen in the autolysed calpain II activity levels among all three experimental groups (Figure 4.5). With 100 µg of soluble protein loaded to the gel (Figure 4.4, Iono), the autolysed calpain II activity levels in the lenses cultured with ionomycin were higher than the other two groups.

Figure 4.5 Casein zymography of calpains present in 25 µg of soluble protein from lenses after culture for 48 h in n EMEM-3.6 (Control), EMEM-3.6 + 5 µM ionomycin (Iono) and EMEM -3.6 + 5 µM ionomycin + 5 mM EGTA (Iono+EGTA). EMEM-3.6 media contains total of 3.6 mM Ca\(^{2+}\). An extract of lung was used as control (Lung). Duplicate zymogram were run for each experimental group.
**Proteolysis in the ionomycin-induced opacity**

The full opacity induced by 48 h ionomycin treatment did not cause gross changes in the lens protein content. There were no significant differences in either the total soluble protein or the urea-soluble protein concentrations among the ionomycin-treated lenses, control lenses or those exposed to EGTA (Table 4.2).

**Table 4.2** Mean protein concentrations (mg/g, ± sd) of whole lenses after culture for 48 h in EMEM-3.6 (Control), EMEM-3.6 + 5 μM ionomycin (Iono) and EMEM -3.6 + 5 μM ionomycin + 5 mM EGTA (Iono+EGTA). Two-sample T test.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>n</th>
<th>soluble proteins (mg/g)</th>
<th>urea soluble proteins (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>555 ± 112</td>
<td>8.9 ± 2.4</td>
</tr>
<tr>
<td>Iono</td>
<td>6</td>
<td>492 ± 104</td>
<td>6.6 ± 2.0</td>
</tr>
<tr>
<td>Iono+EGTA</td>
<td>6</td>
<td>682 ± 143</td>
<td>8.1 ± 0.8</td>
</tr>
</tbody>
</table>

**Cytoskeletal protein degradation**

The cytoskeletal proteins, spectrin, filensin and vimentin, were analysed to examine the degree of their degradation in the OLCO model. The urea-soluble protein separated by SDS-PAGE (Figure 4.6) showed there was complete degradation of spectrin from the ionomycin-treated lenses, whilst spectrin (280 kDa) remained intact in urea-soluble protein from the EMEM control and EGTA control lenses.

**Figure 4.6** SDS-PAGE analysis of the urea soluble protein from ovine lens (12 μg). Lane 1 indicates molecular marker (M) (Precision Plus Protein Standards™, All Blue, Bio-Rad, U.S.A.). Lanes 2, 3 and 4 are lenses after culture for 48 h in EMEM-3.6 (C), EMEM-3.6 + 5 μM ionomycin (I) and EMEM-3.6 + 5 μM ionomycin + 5 mM EGTA (IE) respectively.
There was evidence of partial degradation of the lens specific intermediate filament, filensin (115 kDa, identified in urea soluble fractions extracted from bovine lenses by Marcantonio and Sanderson, 1997) in ionomycin treated lenses, whilst the intensity of the filensin band was stronger in samples from control or EGTA treated lenses.

Another cytoskeletal protein, vimentin was immunoblotted using the urea soluble protein fraction of all three experimental groups (Figure 4.7). Lenses from the control and EGTA-calcium chelated group contained intact vimentin. The lenses from the ionomycin treatment showed that intact vimentin (56 kDa) was no longer present in the urea soluble fraction.

![Figure 4.7 Western detection of urea soluble vimentin from ovine lens proteins (150 μg) after culture in EMEM-3.6 (Control), EMEM-3.6 + 5 μM ionomycin (Iono) and EMEM-3.6 + 5 mM EGTA+5 μM ionomycin (Iono+EGTA) for 48 h. Solid arrow represents intact vimentin (56 kDa).](image)

**Crystallin degradation**

Detailed crystallin proteolysis was analysed by 2-DE. The 2-DE analysis showed that some degree of degradation of soluble proteins occurred in lenses treated with ionomycin (Iono) compared to the control and Iono+EGTA treatment groups (Figure 4.8).

The βA3 and the βA4 crystallins appeared to be reduced in lenses cultured with ionomycin compared to ones in control or EGTA treated lenses. α-Crystallin spots from normal and ionomycin treated lenses were not apparently different. The same spot from EGTA was small due to poor separation. Other notable 2-DE spots (oval on Figure 4.8) appeared at around 65-70 kDa molecular weights in all the three treatment groups. It is, however, more intensive from lenses treated with ionomycin compared to the other two treatment groups.
Overall the 2-DE protein profile of whole lens revealed that the majority of the crystallins in ionomycin-treated lens remained intact and similar to the control and EGTA control lenses.

Figure 4.8 2-DE protein profiles from 75 μg soluble protein extracted from ovine lenses after culture in EMEM-3.6 (Control), EMEM-3.6 + 5 μM ionomycin (Iono) and EMEM-3.6 + 5 μM ionomycin + Ca²⁺ chelator (Iono+EGTA). Molecular marker (Precision Plus Protein Standards™, All Blue, Bio-RAD) was run along with the sample (displayed here on a Control gel). βA3 and βA4 crystallin are marked within rectangles. 2-DE spots for phosphorylated αA-crystallins (αA-ph), intact αA-crystallins (αA) and vimentin are indicated. 2-DE spots for possible cross-linked proteins are also marked within ovals.

4.4. Experiment 2: Progression of opacity in intact ovine lens by ionomycin

The objective of this experiment was to visually characterise the progression of opacity in the lenses from the ionomycin-induced Ca²⁺ overloading system with a lower concentration of ionomycin (1 μM) in the normal culture medium over a longer period of culture time.
4.4.1. Methods

_Lens sample collection and culture preparation_
Lenses were dissected from slaughtered sheep (9-12 months old) and placed immediately in EMEM for a 2 day settling period (refer to the culture protocol, Chapter 3).

_Experimental protocol_
A group of 6 lenses were continuously cultured in the EMEM containing 1.8 mM Ca$^{2+}$ (EMEM), while a second group of 6 were cultured in the EMEM with 1 μM ionomycin for a 7 day experimental period. All lenses were replaced with a fresh mixture every 24 h. The progress of opacification was monitored daily during the experimental period. At the end of 7 days, all lenses were removed from the medium and detached from any adhering non-lens tissues and vitreous humour. The cleaned lenses were then weighed and their wet weights were recorded.

4.4.2. Results

_Monitoring progress of lens opacification_
The progress of opacification was recorded daily on all lenses cultured in normal EMEM and in EMEM with ionomycin (Figure 4.9). Lenses cultured in EMEM remained transparent during the experimental period of 7 days.

Lenses (total of 6) cultured with ionomycin developed opacification with a similar pattern over the experimental period- the progress of visual light scattering starting from the equatorial region. Light scatter was initially apparent in the outermost cortical fiber cells in the equatorial regions of the lens (Day 2). The degree of opacity was greater in the equatorial regions and the opacification progressed towards the center of anterior surface of the lens (Day 3, Day 4 and Day 5). The centre of the anterior region of the lens remained partially transparent.

The lens developed full opacification and stayed opaque by Day 6 and Day 7. At Day 7, during removal of lenses from the culture system, it was noted again that fully opaque lenses were swollen and contained slight pink pigments, as seen earlier (Figure 4.2).
4.5. Experiment 3: Change of calpain II activity levels

The objective of this experiment was to monitor calpain II activity levels in lenses during the gradual development of opacity due to ionomycin.

4.5.1. Methods

**Ovine lens collection and the culture preparation**
A group of ovine eye lenses (unknown age, mixture of breeds) were dissected and cultured in EMEM within 2 h of the death of the animal (refer to the culture protocol, Chapter 3).
Experiment protocol

After 2 days of a lens culture pre-settling period, groups of 3 lenses were cultured with 1 μM ionomycin for a total experimental period of 68 h. The treatment solution of all lenses was replaced daily during the experiment. Every 24 h, all the lenses were harvested and stored at -80°C.

Calpain activity analysis by casein zymography

The epithelium (containing lens capsule) and the cortex of the lens were dissected, and homogenised with 6 volumes of ice cold Buffer-C (20 mM Tris-HCl, pH 7.5; 1 mM EDTA; 1 mM EGTA; 2 mM DTT). These homogenates were centrifuged in Beckman J2-MI centrifuge for 30 min at 48,500 x g (4°C). The supernatant were collected as soluble protein. The soluble proteins from each fraction were run in the casein zymogram to examine the activity level of each calpain isoform, particularly calpain II (refer to Experiment 1, 4.2.1).

4.5.2. Results

It was observed that the opacification were restricted to cortex of the ovine lens (including the epithelium), whilst the nucleus remained transparent. Calpain II activity were examined on the cortex of the lens. Calpain activity of the cortex of the ovine lens (including the epithelium) from both non-cultured lenses (C1) and cultured lenses (C2) for approximately 5 days (2 days pre-incubation + 68 h) were compared by zymography assay (Figure 4.10).

![Figure 4.10 Casein zymography of calpains present in 25 μg of soluble protein from the cortex of the ovine lens (including epithelium) harvested at 24, 44 and 68 h after culture in EMEM+ 1 μM ionomycin. Calpain I (μC) and calpain II (mC) were extracted from sheep lung. Non-cultured lenses (C1) and non-treated control lenses in EMEM for 68 h (C2) were used as controls. Lenses exposed in 1 μM ionomycin for 24 h, 44 h and 68 h are labelled as 24 h, 44 h and 68 h.](image)
There was no enzymatic activity level difference between the two control groups (Figure 4.10, μC and mC), suggesting the lens culture system for the total of 5 days did not influence the calpain activity level of the intact lenses.

The change in calpain activity level during progression of the ionomycin-induced opacification revealed a decrease in intact calpain II activity of ionomycin treated lenses during the 68 h experiment (Figure 4.10). Autolysed calpain II became more abundant as the opacification progressed.

4.6. Experiment 4: Cytotoxicity and morphological changes in the OLCO models.

The objective of this experiment was to investigate cytotoxicity level in the ionomycin-induced opaque lenses and to determine if there was a correlation between the effect of ionomycin-induced Ca\(^{2+}\) influx and the loss of lens transparency.

Difference in cytotoxicity (LDH leakage assay) between control lenses and Ca\(^{2+}\)-chelated lenses were first established to determine the effect of ionomycin without any Ca\(^{2+}\) involvement. Then difference in cytotoxicity levels between ionomycin-treated lenses and Ca\(^{2+}\)-chelated lenses were examined to test the effect of Ca\(^{2+}\) influx into the lenses and the relationship with the progression of lens opacification.

4.6.1. Methods

Lens sample collection & culture preparation
A group of eye lenses from sheep (9 – 11 months old) were dissected and cultured in a normal culture medium, EMEM (refer to the culture protocol, Chapter 3).

Preparation of treatment solution
Ionomycin was solubilized in 2 mg/mL DMSO, and mixed with a normal EMEM (containing 1.8 mM Ca\(^{2+}\)) or EMEM + 1.8 mM Ca\(^{2+}\) (containing 3.6 mM Ca\(^{2+}\)) to make a final ionomycin concentration of 5 μM. For the Ca\(^{2+}\) chelated conditions, 5mM of EGTA was added to EMEM under sterile conditions.
**Experimental protocol**

After 2 days of lens culture pre-settling period, groups of 5 or 6 lenses were cultured for 44 h under four experimental conditions: EMEM (Control); EMEM + 5 μM ionomycin (Iono-1.8); EMEM + 1.8 mM Ca²⁺ + 5 μM ionomycin (Iono-3.6); EMEM + 5 μM ionomycin + 5 mM EGTA (Iono+EGTA). Lenses in the Iono+EGTA group were pre-cultured in 5 mM EGTA for 30 min to chelate external Ca²⁺ before 5 μM ionomycin was introduced. Lenses in Iono-1.8 and Iono-3.6 groups were paired, while Control and Iono+EGTA groups were unpaired.

At the end of 44 h treatment period, all lenses were rinsed with dH₂O and removed from the medium and any adhering non-lens tissue and vitreous humour. The wet weights were determined and the lenses immediately frozen and stored at -80°C. Lens transparency was monitored over the experimental period.

**Ca²⁺ assay by atomic absorption analysis**

Whole lenses were dissected into different parts and subjected to freeze drying. Dry weights were recorded. Nitric acid (69%, Aristar, 10 mL) was added to each sample and mixed thoroughly before sonication for 45 min (Elma Transonic T460 sonic bath) and overnight digestion. The samples were analysed without further dilution on a GBC Avanta atomic absorption spectrophotometer using emission mode plus a C₂H₂/N₂O gas mixture. Standards were made from Spectrosol calcium nitrate AAS standard (1000 ppm) and double deionised water acidified with HNO₃. Data were analysed by ANOVA and then individual treatments were compared by Students t-test.

**Cell viability assay with a cytotoxicity detection kit.**

Aliquots of culture medium were taken from all experimental groups at 30 min, 1 h, 2 h, 4 h, 7 h and 44 h after exposure to the treatment solution and stored at 2 - 5°C for LDH leakage assay (cytotoxicity assay). The details of the procedure are in Chapter 3 (2.2. Method).

**Microscopic view of ionomycin-treated lenses**

Lenses harvested from treatment were washed with dH₂O and each lens was immediately placed in approximately 10 mL of 4% paraformaldehyde in 1 x PBS for 6 days cell fixation. The lenses were then embedded in paraffin, sectioned through the equator, and processed for standard histological staining (Haematoxylin and Eosin stain) as described by Windle (1976).
Nuclei are stained in dark purple/blue, while cytoplasmic components of cells are bright pink/light red. The stained cell slices were examined under a light microscope (Leica Microscope, model No. 2.2.1, Diagnostic Instrument Inc., U.S.A.).

*Lens opacity grade scoring by image analysis system*
A digital camera was set directly above the anterior epithelium of the lens. Opacification of the lens was graded as described in Chapter 3 (2.2. Method).

### 4.6.2. Results

*Cytotoxicity and lens transparency*
This experiment demonstrated that cytotoxicity occurred in the lenses treated with ionomycin and Ca\(^{2+}\) containing medium (Figure 4.11). Within the first 2 h of the experimental period, the LDH leakage levels from ionomycin-treated lenses (1-1.8 and I-3.6) increased and were significantly higher than the lenses treated with ionomycin in the presence of a Ca\(^{2+}\) chelator (El) (P < 0.02) (Figure 4.11). Over the next 24 h experimental period, the difference in the LDH leakage level between ionomycin-treated (I -1.8 and I-3.6) and Ca\(^{2+}\) chelated (El) groups increased dramatically (7 fold). During this experimental period, LDH leakage levels of the Ca\(^{2+}\) chelated (El) group remained as low as the control group lenses (Control) (Figure 4.11). Chelating the external Ca\(^{2+}\) clearly prevents the occurrence of cytotoxicity. These data also indicate that ionomycin itself does not cause cytotoxicity.

Ca\(^{2+}\) chelated lenses (Iono+EGTA) showed a minor degree of light scattering at the early stage of incubation (Figure 4.12). There was, however, no sign of cytotoxicity with the 24 h treatment. The LDH leakage (5.29 ± 2.63 mU/mL) of Iono+EGTA was increased at the end of the 44 h experimental period compared with control lenses (1.32 ± 1.1mU/mL), but was still low compared to the LDH leakage in ionomycin treated lenses cultured for 44 h (average 37.5 ± 3.5 mU/mL). With 5 μM concentration of ionomycin, the progress of cytotoxic level in the lenses was not significantly different between the two external Ca\(^{2+}\) environments, 1.8 mM and 3.6 mM Ca\(^{2+}\), over the entire experimental period. The progress of opacification in the ionomycin treated lenses was also similar between these two groups (Figure 4.12).
Figure 4.11 A plot of mean LDH leakage levels detected in 4 lens experimental groups over a total 44 h experimental period. Lenses were cultured in EMEM (Control), EMEM + 5mM EGTA + 5 μM ionomycin (EI), EMEM + 5 μM ionomycin (I-1.8) and EMEM + 1.8 mM Ca$^{2+}$ + 5 μM ionomycin (I-3.6). EMEM + 1.8 mM Ca$^{2+}$ contains a total of 3.6 mM Ca$^{2+}$ whilst EMEM contains a total of 1.8 mM Ca$^{2+}$.

Figure 4.12 A plot of mean opacification scores (%: 100 is fully opaque, 1 is transparent lens) for 4 lens experimental groups over total 44 h experimental period. Lenses were cultured in EMEM (Control), EMEM + 5mM EGTA + 5 μM ionomycin (Iono +EGTA), EMEM + 5 μM ionomycin (Iono-1.8) and EMEM + 1.8 mM Ca$^{2+}$ + 5 μM ionomycin (Iono-3.6).
Changes in the ion contents as determined by atomic absorption analysis

Total Ca\(^{2+}\), Na\(^+\) and K\(^+\) levels in the lens were measured by atomic absorption analysis and compared between normal (Control), ionomycin treated (Iono-1.8) and ionomycin with Ca\(^{2+}\) chelator treated (Iono+EGTA) groups of lenses (Table 4.3).

The absence of Ca\(^{2+}\) in the external environment resulted in changes in the cellular concentration of all three ions, compared to control lenses. The Na\(^+\) content of the lenses approximately doubled on reducing the external Ca\(^{2+}\) using the Ca\(^{2+}\) chelating agent, EGTA, in an ionomycin containing media (Iono+EGTA). The K\(^+\) level of the lenses in the Iono+EGTA treated group was reduced by approximately 50% compared with the control lenses. There were no significant changes in the mean wet weight of the lens from the Iono+EGTA treated group compared to control lenses after a 44 h experimental period.

In contrast, opaque lenses in the ionomycin treated group were approximately 10 fold higher in their total mean Ca\(^{2+}\) level compared to the control lenses. The Na\(^+\) level increased by 4.5 fold in the lenses treated with ionomycin compared to the control lenses. The K\(^+\) level of the lenses in the ionomycin treated group was reduced by approximately 10% compared with the control lenses.

Lenses from the ionomycin treated group showed a 10-16% increase in their mean wet weights compared to the control and EGTA treated groups (P < 0.01).

Table 4.3 Mean total ion concentration (mM) and wet weight of whole ovine lens (g, ± s.d) after culture in EMEM (Control), EMEM+5 μM ionomycin + 5 mM EGTA (Iono+EGTA) and EMEM + 5 μM ionomycin (Iono-1.8).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>wet weight (g)</th>
<th>Ca(^{2+}) (mM)</th>
<th>Na(^+) (mM)</th>
<th>K(^+) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>1.016 ± 0.08</td>
<td>0.37 (± 0.05)</td>
<td>19.3 (± 4.5)</td>
<td>47.6 (± 4.6)</td>
</tr>
<tr>
<td>Iono+EGTA</td>
<td>4</td>
<td>1.086 ± 0.04</td>
<td>b0.13 (± 0.03)</td>
<td>v43.3 (± 7.5)</td>
<td>x26.3 (± 3.5)</td>
</tr>
<tr>
<td>Iono-1.8</td>
<td>3</td>
<td>a1.181 ± 0.07</td>
<td>c3.03 (± 0.15)</td>
<td>y90.3 (± 5.4)</td>
<td>xx36.3 (± 2.0)</td>
</tr>
</tbody>
</table>

Two-sample T test;

\( ^{a}P < 0.01 \) relative to both Control and Iono+EGTA.

\( ^{b}P < 0.001 \) relative to Control, \( ^{c}P < 0.001 \) relative to Control.

\( ^{v}P < 0.005 \) relative to Control, \( ^{x}P < 0.001 \) relative to Iono+EGTA.

\( ^{y}P < 0.001 \) relative to Control, \( ^{xx}P < 0.01 \) relative to Iono+EGTA.

The major feature of the OLCO model was the appearance of a large volume of turbid liquid located between the lens capsule and inner cortex region. This liquid was restricted to the intact lens capsule and was isolated from the remainder of the lens (nucleus region), which
retained its gel-like structure and maintained its transparency. The disintegrated fiber cell fraction is the most likely the source of light scattering in the OLCO model.

Analysis of the Ca\(^{2+}\) concentration in the liquid fraction revealed that this fraction was largely responsible for the increased Ca\(^{2+}\) levels in the whole lens (Table 4.4). When two groups of lenses were exposed to the same ionomycin concentration, but two different external Ca\(^{2+}\) concentrations, the mean Ca\(^{2+}\) concentration of the liquid fraction in the lenses increased by 150 - 200\% compared to the external Ca\(^{2+}\) concentration in the culture medium. The rise of lens Ca\(^{2+}\) level is, therefore, actively induced by the presence of ionomycin in the medium and is not a result of an ion equilibrium between the medium and lens.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Ca(^{2+}) (mM) in the culture medium</th>
<th>Liquefied fraction (mM)</th>
<th>Inner most cortex + nucleus region (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iono-1.8</td>
<td>3</td>
<td>1.8</td>
<td>4.29 ± 0.32</td>
<td>1.22 ± 0.10</td>
</tr>
<tr>
<td>Iono-3.6</td>
<td>3</td>
<td>3.6</td>
<td>8.06 ± 0.24</td>
<td>2.03 ± 0.15</td>
</tr>
</tbody>
</table>

Two-sample T test. *P* < 0.001 relative to Iono-1.8, **P** < 0.005 relative to Iono-1.8

**Morphological feature**

A cross section of the ovine lens was stained with Haematoxylin and Eosin to examine morphological features in the OLCO model. Pink/light-red is the staining colour for the cytoplasmic components. The background colour is light green/yellow. Any artificial cracks created during fixation or the paraffin slicing preparation are indicated by black arrows in the Figures 4.13 by. Black/dark grey dot on the lens surface represent nuclei of epithelial cells, and of fiber cells that still contain nuclei (Figure 4.13, A).

In control lenses cultured in EMEM for 2 days (A), there was no sign of structural breakage between the epithelial cells and the rest of fiber cells. In these lenses, staining was consistent and uniform across the cells. When lenses were cultured in a Ca\(^{2+}\) chelating media containing ionomycin, some fiber cells very near the surface displayed enlarged extracellular spaces.
The rest of the fiber cells showed consistent and uniform staining across the cells.

The microscopic views of the fully opaque lens showed that the structural integrity was no longer present in the outer cortex region (Figure 4.13, B). A cluster of some cytoplasmic components (red staining) was randomly mixed with discrete areas (grey staining in circular shapes, indicated by white arrows on Figure 4.13, B) and empty spaces. Interestingly, this region is distinctively separated from the inner region of the lens, where the fiber cells (inner fiber cells, Figure 4.13, B) display different structural feature.

\textit{capsule + epithelial cells (nucleus in dark dots)}

![Figure 4.13](image)

\textbf{Figure 4.13} Microscopic views of ovine lens after culture in (A) EMEM (Control), (B) EMEM + 1.8 mM Ca\textsuperscript{2+} + 5 μM ionomycin (Iono-3.6) and (C) EMEM + 1.8 mM Ca\textsuperscript{2+} + 5 μM ionomycin + 5mM EGTA (Iono-3.6 +EGTA) for 44 h. Artificial cracks marked by black arrows (A and C).
4.7. Discussion

**Opacification in the OLCO model was associated with Ca\(^{2+}\) overload**

Alteration in Ca\(^{2+}\) homeostasis influences the overall ionic homeostasis in the lens. Current data showed that both ionomycin and ionomycin-EGTA treatments induced the loss of K\(^{+}\), Na\(^{+}\) and Ca\(^{2+}\) balance in the lens (Table 4.3). The features of lenses cultured with ionomycin and Ca\(^{2+}\) (i.e. dense opacity and lens hydration) were largely prevented by chelation of the Ca\(^{2+}\). Total Ca\(^{2+}\) level assays showed that the opaque regions of the lens were strongly correlated with the elevation of Ca\(^{2+}\) (Table 4.4). That is, increases in intracellular Ca\(^{2+}\) level are strongly associated with development of opacity, which is the characteristic of the OLCO model.

The application of the ion selective micro electrode technique by other researchers has demonstrated that post extraction cortical human cataract lenses increased their free level of Ca\(^{2+}\) which equilibrated with the aqueous humour (1–2 mM). The total Ca\(^{2+}\) of cataractous lenses could increase beyond 20 mM (Duncan & Jacob, 1984). Truscott et al. (1990) also demonstrated that there was a direct linear relationship between an increase in internal free Ca\(^{2+}\) and an increase in cortical opacification.

In an earlier study with cultured bovine lens, Marcantonio et al. (1986) demonstrated that lens hydration was independent of opacification and that an increase in lens intracellular Ca\(^{2+}\) was crucial for opacification. Opaque lenses with major swelling were observed in ionomycin-induced opacification in rat (Iwasaki et al., 1995; Sanderson et al., 1996) and human (Sanderson et al., 2000) lenses and in advanced human *in vivo* cortical cataracts (Heyningen, 1972). The increase of lens hydration in the OLCO model in this thesis may be an effect of opacification.

Current data reveal that the total Ca\(^{2+}\) concentration of normal cultured ovine lens (1.8 mM Ca\(^{2+}\) in EMEM) was approximately 370 \(\mu\)M Ca\(^{2+}\) (Table 4.3). Total Ca\(^{2+}\) concentrations determined by atomic absorption techniques in bovine (Marcantonio et al., 1986) and rat (Duncan & Jacob, 1984) lenses showed 500 \(\mu\)M and 670 \(\mu\)M Ca\(^{2+}\) respectively. In the current study, when the lenses were exposed to a high concentration of external Ca\(^{2+}\) in the medium (3.8 mM Ca\(^{2+}\) in EMEM), the total lens Ca\(^{2+}\) concentration was increased (Table 4.1) but lens transparency remained intact over a 4 day incubation period (2 day settling period and 2 day
experimental period) (Figure 4.2). This result demonstrates the ability of the lens to sustain
its Ca\(^{2+}\) homeostasis despite a two fold increase Ca\(^{2+}\) in the external aqueous environment. In
clear human lens, lens lipids show a capacity to bind Ca\(^{2+}\) (Tang et al., 2003). There are
proteins that act as buffers for Ca\(^{2+}\) (Louis et al., 1986) and there are internal compartments
for sequestering Ca\(^{2+}\) (see Table 1.1) (Clapham, 1995; Duncan et al., 1994). It is documented
that over 99% of total Ca\(^{2+}\) in the lens is in the bound form and that less than 1% is free
(Duncan et al., 1989; van Marle et al., 1997; Vrensen et al., 1995).

**Ca\(^{2+}\) influx by ionomycin and lens physiological zones.**
The microcirculatory system of the lens drives the flow of fluid. The assumption is that
ionomycin follows the flow and enters the lens through extracellular spaces and then reach the
core of the lens. There is a considerable amount of Ca\(^{2+}\) located in the intercellular spaces of
human and rat lenses (van Marle et al., 1997; Vrensen et al., 1995). In the present work with
young cultured ovine lenses (6-9 months old) the effect of adding ionomycin to the medium
was restricted to the development of opacity in the cortex, which was associated with an
increase in Ca\(^{2+}\) concentration within this region.

Early reports with a Ca\(^{2+}\) ionophore showed that there was limited complexing of Ca\(^{2+}\) ions
with the Ca\(^{2+}\) ionophore below pH 7.0 (Liu & Hermann, 1978). Transmembrane pH gradients
also alter the transport properties of Ca\(^{2+}\) ionophores (Erdahl et al., 1995; Wolley et al.,
1995). The inner fiber cells (core of the lens) provide an acidic environment (Mathias et al.,
1991). This acidity may limit the response to a Ca\(^{2+}\) influx action in the core of the lens
induced by ionomycin. In other tissues, ionomycin also enhances Ca\(^{2+}\) influx by stimulating
store-regulated cation entry (Dedkova et al., 2000; Morgan & Jacob, 1994). Internal Ca\(^{2+}\)
regulatory compartments, such as the ER and mitochondria, are completely removed during
terminal differentiation of fiber cells and they are absent in the inner fiber cells. Hence, the
influx of Ca\(^{2+}\) induced by ionomycin would be limited at the cortical region of the lens which
contain differentiating fiber cells.

The cellular response to cataractogenetic insults may be also different between the two
physiological zones. Current Ca\(^{2+}\) data (Table 4.4) show that the nucleus region had a certain
degree of Ca\(^{2+}\) influx but still remained transparent, unlike the cortical region which had a
large influx of Ca\(^{2+}\) and exhibited dense opacity. Adult rat lenses studied by Iwasaki et al.
(1995) demonstrated there were large increases in the concentration of insoluble protein in the nuclear region that remained transparent under Ca\textsuperscript{2+} ionophore treatment. Thus, the mechanism of opacity development may differ between the cortex and nucleus of the lens.

The susceptibility of different regions of rat lenses to a cataractogenetic agent also changed with increasing age. With ionomycin exposure, young rat lenses (less than 4 weeks old) developed dense nuclear opacity (Azuma et al., 1992; Fukiage et al., 1997b; Iwasaki et al., 1995; Nakamura et al., 2003) but adult rat lenses (12 weeks old) developed cortical cataracts (Iwasaki et al., 1995; Sanderson et al., 1996). Young rats (less than 1 week old) injected with sodium selenite (selenite cataract, refer to Chapter 1) developed dense nuclear opacity and Ca\textsuperscript{2+} accumulated in the lens nucleus (Shearer et al., 1997). On the other hand, human cortical cataractous lenses displayed changes in their internal ionic contents, especially a large increase in Ca\textsuperscript{2+}, whilst nuclear cataracts showed no changes in internal ionic content (Duncan & Bushell, 1975; Duncan & Jacob, 1984). Such research indicates that there are functional and physiological differences between cortex and nucleus regions of the lens and the opacity development.

The cause of the increase in internal Na\textsuperscript{+} level in ionomycin-EGTA treated lenses may be due to the opening of a non-selective cation channel in response to the lowering internal Ca\textsuperscript{2+} level (Rae et al., 1992).

*No loss of lens dry weight was observed in the OLCO model*

Early reports with mature human cortical cataractous lenses revealed that the degree of disruption in the ionic balance correlated with the loss of protein and, as a consequence, a loss in the dry weight of the cataractous lens. Duncan and Bushell (1975) showed that an increase in the cytosolic Ca\textsuperscript{2+} is associated with a decrease in the lens dry weight. Recent human lens culture studies (Sanderson et al., 2000) also showed there was a strong link between increased Ca\textsuperscript{2+} levels and the loss of newly synthesized proteins. However, in ovine lenses both the protein contents (urea soluble and total soluble protein contents) and the dry weights of the opaque lenses treated with ionomycin were not significantly different from the control and EGTA-treated lenses (Table 4.1 and 4.2).
One possible explanation for the difference between these data and others could be the rapid progress of opacification in the OLCO model (i.e. 2 - 3 days) due to the traumatic Ca\(^{2+}\) influx through the action of ionomycin, compared to several months, as observed in cataract formation under *in vivo* conditions (Robertson *et al.*, 2005). The bovine lens culture system demonstrated there was a catastrophic differential loss of lens proteins, which was only apparent after 6 days. This was associated with an increase in lens Ca\(^{2+}\). No protein loss was observed in the EGTA treated lenses over 10 day experimental period (Marcantonio *et al.*, 1986).

**Ca\(^{2+}\)-activated proteolysis was associated with the opacification**

In the cultured ovine lenses, the maintenance of a lens gel state is more dependent on internal Ca\(^{2+}\) than any other cation (Table 4.3 and 4.4). Elevation of Ca\(^{2+}\) in the OLCO model induced opacification of the lens. Microscopic cross sections of the opaque lenses revealed a turbid fraction, which contained totally disintegrated structures. This fraction readily segregated from the rest of the fiber cells. The latter showed no signs of structural disintegration (Figure 4.13). The formation of turbidity in the outer shell fiber cells was a result of high Ca\(^{2+}\)-induced biochemical and morphological changes. The detrimental effects from the loss of Ca\(^{2+}\) regulation by the lens illustrate the importance of Ca\(^{2+}\) homeostasis in this region of the lens. It is speculated that the loss of Ca\(^{2+}\) regulation in the outer shell fiber cells may trigger initial cellular changes and, as a consequence, the cell membrane integrity is disturbed which leads to an increase in cell permeability. This results in the loss of water homeostasis and cellular ionic homeostasis and, consequently, further membrane degradation. Bettelheim (1983) observed morphological degeneration and disintegration of membranes in many cataractous processes. The higher Ca\(^{2+}\) levels in the outer shell region compared to the inner fiber cells are, therefore, a result of the initial Ca\(^{2+}\)-driven changes.

It is a prominent feature of the OLCO model that the equatorial region of the lens is the initial site for opacity (Figure 4.9) and that the cytoskeletal proteins, vimentin and spectrin, are subjected to degradation in the opacification process (Figure 4.6 and 4.7). The equatorial region of the lens is composed of young differentiating fiber cells that are enriched with cytoskeletal proteins, which assist the differentiation process of the epithelial cells into fiber cells (Bassnett & Beebe, 1992; Goodenough, 1992; Prescott *et al.*, 1994). Proteolytic modification is an important process which occurs during normal development of lens
transparency. This proteolytic process is tightly regulated under normal physiological conditions. The data in this thesis show that calpain II is the dominant calpain isoform present in the lens (Figure 4.4). The correlation of calpain II activity with the progress of opacification (Figure 4.4 and 4.10) indicated that there is a possible linkage between the involvement of calpain II and the rapid protein degradation in the outer shell region. Most calpain II activity is detected in the outer shell region and there is little calpain II in the nuclear region in bovine (Yoshida et al., 1985) and ovine lenses (Robertson, 2003).

Many studies have proposed that the degradation of proteins, which are usually involved in the maintenance of lens transparency, are induced by over-activation of the Ca\(^{2+}\)-dependent proteases or calpains (David & Shearer, 1986, 1993; David et al., 1993; Shoeman & Traub, 1990; Yoshida et al., 1984). Indeed, some cytoskeletal proteins are calpain substrates. Spectrin and vimentin, in particular, are rapidly degraded by calpain (Goll et al., 2003; Shoeman & Traub, 1990). Ca\(^{2+}\)-induced degradation of lens cytoskeleton proteins has been reported in lenses cultured from rats (Sanderson et al., 1996; Vrensen et al., 1995), cattle (Marcantonio & Duncan, 1991) and humans (Sanderson et al., 2000). Protein degradation was correlated with the loss of lens transparency.

Taken together, the Ca\(^{2+}\)-induced lens opacification in the OLCO model described in this thesis is associated with proteolysis. The data support the involvement of the calpain-activated proteolytic activity, which may initiate the development of opacification.

**Crystallin degradation contributed a minor factor in the OLCO model**

The aggregation and high concentration of partially unfolded proteins lead to the formation of the insoluble protein characteristics of *in vivo* cataractous lenses (Harding, 2002). There is extensive evidence that the transparency of lens is largely determined by the properties of crystallins. Crystallins are the major lens structural proteins. Limited hydrolysis of the crystallins by calpain contributes to the light-scattering characteristics of cataractous lenses (Augusteyn, 2004). It is, however, surprising in this thesis that SDS-PAGE protein profiles showed no evidence of fragmented crystallins previously identified in opaque lens (Figure 4.6). The 2-DE profiles showed there were only limited modification of some \(\beta\)-crystallin subunits and no apparent modification of any \(\alpha\)-crystallins (Figure 4.8), despite the dense opacity in the OLCO model.
The reduced content of the β-crystallin subunits, βA3 and βA4, in the OLCO model provided some evidence of partial β-crystallin degradation (Figure 4.8). Truncation of the N-termini of β-crystallins by calpain II specific proteolysis has been identified in rat lens (David et al., 1992) and unregulated proteolysis causes opacification (David et al., 1994a). β-Crystallins are rapidly expressed during cell elongation in young differentiating fiber cells at the equatorial region of the lens (Goodenough, 1992). Crystallin modification occurs as part of the normal maturation processes and it was suggested that the site specific proteolysis of β-crystallins may serve to initiate tighter packing of crystallins during lens maturation in young lenses (Werten et al., 1999).

α-Crystallins prevent the precipitation of rat crystallins truncated by proteolysis (Shearer et al., 1995). Similarly, proteolytic products of human and bovine crystallins are not precipitated in the presence of α-crystallins (Shih et al., 2001). Truncation of the C-terminal of α-crystallins by calpain is known, however, to cause the loss of its chaperone function (Kelley et al., 1993; Takemoto, 1994) and consequently α-crystallin fragments entangle in the resulting insoluble pellet as reported for in vitro cataract models (Horwitz, 2003). In 2-DE protein profiles (Figure 4.8), examination of the soluble fraction of the lens protein revealed that intact and phosphorylated forms of αA-crystallin were prominent proteins in the normal lens, but they showed no evidence of degradation in opaque lenses. Partial degraded β-crystallins with undegraded αA-crystallin may make little contribution to the light scattering in the OLCO model.

A recent study, however, demonstrated the deleterious effect of high Ca\(^{2+}\) concentrations upon the chaperone function of α-crystallins without truncation (del Valle et al., 2002). Ca\(^{2+}\) decreased the structural stability of α-crystallins by promoting the partial unfolding of the protein and, as a consequence, impaired the chaperone function and lowered the protective ability of other lens proteins. Thus, the modification of α-crystallins by high Ca\(^{2+}\), without cleavage, could have resulted in the intact crystallin profiles shown on both SDS-PAGE and 2-DE electrophoresis analysis in the data.

The lack of significant changes in the total protein content and dry weight in the OLCO models (Table 4.2 and 4.3) supports the proposal that modification of the crystallins made only a minor contribution to the opacification under this dramatic alteration of Ca\(^{2+}\) homeostasis. Studies by Truscott et al. (1990) with rat lenses showed that a rapid rise of free
Ca\textsuperscript{2+} was associated with a rapid increase in opacification, a massive degradation of the lens cytoskeletal proteins and minor crystallin degradation.

**Role of cytoskeletal elements and the maintenance of the lens transparency**

The major finding of this research is that the initial calpain proteolysis of cytoskeletal elements is a key factor in cortical opacity in the OLCO model. Bettelheim (1985) proposed that lens transparency was physically based on spatial fluctuation in the concentration and optical orientation of macromolecules in the lens, and any changes in the fluctuations in cataractous lens increase light-scattering. For instance, when lens proteins were extracted and a more dilute protein solution prepared than in the normal lens, the protein solution became turbid due to the increased separation of the scattering particles, which cause the destructive scattering associated with low vision (Bettelheim, 1985). According to the physical basis of lens transparency, the formation of turbidity could be due to water uptake leading to diluted lens proteins (composed of limited modified crystallins). The water uptake may be a result of the initial destruction of cell membranes by calpain proteolysis of the key cytoskeletal proteins in the differentiating region of the lens.

Current data support the significance of cytoskeletal proteins in the lens transparency. The importance of interactions between cytostructural proteins and crystallins in the lens was proposed by Clark *et al.* (1999). These interactions are required for the initial organisation of lens proteins into the transparent homogeneous structure, which is necessary to provide an environment favourable for transparency in normal mammalian lens, especially within the cell differentiating zone (Clark *et al.*, 1999). In the absence of a cytoskeletal scaffold, attractive interactions between lens proteins may dominate and accelerate the organisation of proteins into large light-scattering aggregates without modification of the crystallins themselves. When the lens are exposed to a high external Ca\textsuperscript{2+} environment, the degradation of cytoskeleton proteins may result in aggregation of crystallins from a single homogenous transparent phase into separate cytoplasmic domains of condensed protein aggregates, which are responsible for the loss of transparency.

**Non-proteolytic evidence: Cross-linking aggregates**

In addition to the presence of limited proteolysis of crystallins in the OLCO model, the presence of non-proteolytic products was also identified in 2-DE protein profiles of opaque
lenses (Figure 4.8). Elevation of Ca\(^{2+}\) causes cross-linking of cytoplasmic crystallin and membrane proteins to produce the nucleation of large aggregates (Figure 2.1, Chapter 2) (Spector et al., 1979). These large aggregates are not degraded forms of any molecules, but linked at the intra- or inter-molecular level and cause light-scattering in cataractous lens. In ionomycin treated lenses, the intense 2-DE spots around 65 - 70 kDa coincided inversely with the weakening of βA3- and βA4-crystallins spots, suggesting they could be some cross-linked products of the β-crystallin subunits.

Transglutaminase is a Ca\(^{2+}\)-activated lens enzyme that is involved in the cross-linking of lens proteins (Lorand et al., 1981; 1998; Vescio et al., 1990). The activity of transglutaminase has been observed in human lens (Hidasi and Muszbek, 1995). α-Crystallins and vimentin have been reported as substrates for lens transglutaminase (Clement et al., 1998; Shridas et al., 2001). The Ca\(^{2+}\)-dependent cross-linking of vimentin in human lens (Sanderson et al., 2000) suggested a possible role for this enzyme in cataractogenesis. Cross-linking aggregates and proteolysis of proteins can disturb the highly organised spatial orientation of macromolecules, which results in the loss of water homeostasis and so contributes to light scattering.

**Cell death and opacification**

The relationship between opacity and cytotoxicity of the opaque lenses demonstrates that the opacification accompanies cell death in the lens (Figure 4.11 and 4.12). Cell death is associated with elevated Ca\(^{2+}\) level in the lens since the addition of Ca\(^{2+}\) chelating agent to the culture media largely prevented LDH leakage (Figure 4.11).

It is arguable whether cell death, which occurred from the sustained elevation of cytosolic Ca\(^{2+}\) levels, was an effect or cause of cataractogenesis in the OLCO model. For example, when cells are injured by extreme stress or chemical challenges to the point where they are beyond repair, necrotic cell death is associated with massive Na\(^{+}\) and Ca\(^{2+}\) influxes, and consequently calpain activation occurs (Majno & Joris, 1995; Wang, 2000). It is known that calpain activated proteolysis by high intracellular Ca\(^{2+}\) contributes to necrotic cell death in neural pathological diseases like ischemic and excitotoxic neural injury (McGinnis et al., 1999; Wang & Yuen, 1998). An increase in cytoplasmic Ca\(^{2+}\) concentration has also been linked with the initiation of apoptosis in both neuronal and non-neuronal tissue studies (Joseph et al., 1993; McConkey et al., 1989; Pigozzi et al., 2004; Takei & Endo, 1994).
Once a fiber cell loses its physiological homeostasis, cell death could be an inevitable outcome as the lens is a unique structure based on a fine physiological balance. The data in this chapter imply that degradation of lens cytoskeletal proteins results in the formation of the turbidity, which is an outcome of cell death. Conclusively, cataractogenesis leads the lens cells to death. It is likely that the sustained influx of Ca\(^{2+}\) into the lens is involved in necrotic cell death rather than the apoptotic pathway.

When the lens was exposed to the prolonged low levels of intracellular Ca\(^{2+}\) (by EGTA treatment) there was minor cell death. Microscopic examination of the EGTA treated lenses (Figure 4.13) showed that there was some structural modification on the surface of the fiber cells. Vrensen et al. (1995) showed that the lowering of intracellular Ca\(^{2+}\) by addition of EGTA to the extracellular environment induces the formation of large extracellular vacuoles by changes in water homeostasis and Na\(^{+}\) influx. The lowering of intracellular Ca\(^{2+}\) by addition of EGTA may also cause the release of stored Ca\(^{2+}\), which can elicit caspase-3-like protease activity that leads to apoptosis (McGinnis et al., 1999). It is speculated that controlled cell death by the lens, like apoptosis, would involve in removing any unnecessary cells with minimal change in structure integrity. Apoptosis can prevent triggering any major threat to the lens or altering the physiologically balanced status.

**Morphological comparison between the OLCO model and inherited ovine cataracts**

Opacification induced in the OLCO model is initiated from the equatorial region and uniformly develops. In comparison to the OLCO model, the initial stages of *in vivo* inherited ovine cataract is the discrete appearance of opacity alongside clear area in the cortical region (Duncan & Jacob, 1984; Gandolfi et al., 1990; Robertson, 2003). The steady increase in Ca\(^{2+}\) levels in the *in vivo* cataract results in cell uncoupling (Loewenstein, 1981), which is probably due to the occlusion of membrane communicating junctions (Peracchia & Peracchia, 1980). As in other tissues, such uncoupling could act as a protective mechanism, effectively isolating damaged fiber cells from the viable parts of the lens.

However, once the inherited ovine cataract has progressed to a mature stage, this *in vivo* cataract resembles the uniformly dispersed opacity observed in the *in vitro* OLCO models (Robertson et al., 2005). In the *in vivo* cortical cataractogenesis, the progress of opacification
takes up to several months (Robertson et al., 2005). When lenses in the OLCO model were exposed to a rapid increase of ionomycin-induced Ca\textsuperscript{2+}, over a relatively short time period (24 h up to 4 days) or the lenses were exposed to sustained Ca\textsuperscript{2+} for long time period, as in the \textit{in vivo} cortical cataract, the uncoupling mechanisms to protect other fiber cells would still not be sufficient.

4.8. Conclusions

This chapter illustrates some of the biochemical and structural changes which occur in young cultured ovine lens (less than 1 year old) following the rise of intracellular Ca\textsuperscript{2+} induced by ionomycin. The biological and morphological features of the ovine lens calcium-induced opacification (OLCO) system provide a model to understand the role of Ca\textsuperscript{2+} homeostasis in the lens system. The OLCO model demonstrates that cytoskeletal proteins are highly susceptible to degradation under the changes in Ca\textsuperscript{2+} homeostasis. This model supports the importance of cytoskeletal proteins in maintaining lens transparency.

Calpain activation is highly correlated with opacification in this model. Major degradation of known calpain substrates, spectrin and vimentin, are a primary feature of the Ca\textsuperscript{2+} overloaded ovine lens. The nature of the opacification of the OLCO model is the turbid fraction with increased lens hydration in the cortex.

Taken together, these data suggest that deregulation of calpain activity induced by high Ca\textsuperscript{2+} levels could directly trigger major cytoskeletal protein degradation, which is part of a cascade of events leading to further detrimental effects on lens membrane structural integrity impacting on the ionic and water homeostasis of the lens. The loss of Ca\textsuperscript{2+} homeostasis may ultimately lead the lens to cell death and thus cataractogenesis.
Chapter 5: Application of Calpain Inhibitors to Cataractogenesis Models

5.1. Introduction

Understanding the physiological function of calpains during normal lens development and their involvement in cataractogenesis is a challenge for researchers. The application of calpain inhibitors to various tissues is a key tool in determining not only the role of calpains in normal and pathological processes, but also to assess their potential as therapeutic agents. The most specific calpain inhibitor is the endogenous inhibitor of calpain I and calpain II, calpastatin (Murachi, 1983). However, this has limited therapeutic potential, as calpastatin and its active polypeptide fragments are large molecules and have problems crossing membrane barriers.

As a consequence, the development of exogenous calpain inhibitors has focused on designing active-site targeting peptide analogues of calpain substrates with increased membrane permeability for their effectiveness as anti-calpain drugs. Several exogenous calpain inhibitors have been used to investigate the role of calpains in a variety of human diseases, including arthritis (Yamamoto et al., 1992), cerebral and myocardial ischaemia (Wang & Yuen, 1994), muscular dystrophy (Bhattacharyya et al., 1991), Alzheimer's disease (Saido et al., 1993a) and cataract formation (Azuma et al., 1991).

Experimental animal models have also been exposed to exogenous calpain inhibitors to determine the role of calpain as the mechanism of cataractogenesis in the progress of chemically-induced opacification (Azuma et al., 1991, 1992; Fukiage et al., 1997b; Kadoya et al., 1993; Lampi et al., 1992; Sanderson et al., 1996).

SJA6017 (N-(4-fluorophenyl-sulfonyl)-L-valyl-L-leucinal) is an active-site targeting peptide aldehyde calpain inhibitor with better cell permeability characteristics than leupeptin and E64 (Fukiage et al., 1997b). Data show it inhibits calpain I (IC$_{50}$ = 7.5 nM) and cathepsins (IC$_{50}$ <15 nM) better than calpain II (IC$_{50}$ = 78 nM). SJA6017 has no significant effect on the activity of many other cysteine and serine proteases (Inoue et al., 2003). The application of SJA6017 has been shown to reduce the rate of cataract formation in animal models (Biswa et
Despite the remarkable inhibitory effects of these calpain inhibitors in cell-free assay systems, many in vitro and in vivo studies have illustrated that the calpain inhibitors have only partial inhibitory effects on the progress of opacification. Two in vitro Ca\(^{2+}\)-overloading cataractogenesis models, the OLCO model (Chapter 4) and the hyper-Ca\(^{2+}\) medium model (Biswa\textit{ et al.}, 2004; Hightower & Dering, 1984; Hightower & Farnum, 1985), were used in the first part of the current chapter to further investigate the role of calpain in cataractogenesis by the application of the exogenous calpain inhibitor, SJA6017 to the lens culture system. These two culture systems provided variation in the way the opacification developed within the lens. Applying the calpain inhibitor directly to the lens culture system resembles the uptake of the calpain inhibitors from the aqueous humour to cells of the lens in the intact eye. Investigating the efficacy of calpain inhibitors in a lens culture system also indicates their potential as therapeutic agents in in vivo cataractogenesis.

Systemic delivery of the drug to the eye by oral administration or injection can be limited by the relatively poor penetration of some groups of agents into the ocular tissues from the plasma (Freddo, 2001), and can generate potential side effects (Burstein, 1985). Local administration of the drug to the eye has the advantage of achieving relatively high local concentrations with only marginal drug exposure to the rest of the body. Topical administration has been used as a route of local delivery in treating diseases involving the pre-corneal tissues, cornea, iris, and ciliary body (Andermann \textit{et al.}, 1978; Burstein, 1985; Havener, 1983; Lee & Robinson, 1986; Maurice & Mishima, 1984; Piercey, 1985). Topical delivery of anti-cataract agents could be, therefore, the most desirable approach to treating the lens.

The cataract group at Lincoln University and the University of Canterbury, New Zealand, have designed novel anti-calpain compounds based on the structure of SJA6017 (Figure 5.6). Novel calpain inhibitors were tested for their ability to slow the development of induced opacity in the \textit{in vitro} ovine lens system. Then, the \textit{in vivo} assessment involved long term topical administration of selected novel calpain inhibitors to a flock of sheep with naturally developing inherited cataracts.
5.2. Experiment 1: Application of calpain inhibitor in the culture system.

The purpose of this experiment was to confirm the involvement of calpain activity in the mechanism of \( \text{Ca}^{2+} \)-induced cataract formation, by applying a known calpain inhibitor, SJA6017, to the ovine in vitro lens culture system.

Two experiments, experiments 1-a and 1-b, were designed to investigate the effect of adding an inhibitor of calpain to two different cataractogenesis models, the OLCO and hyper-\( \text{Ca}^{2+} \) incubation media models.

5.2.1 Experiment 1-a: Calpain inhibitor in the OLCO model

5.2.1.1 Methods

**Lens culture**
Eye globes were obtained from 11-month-old sheep immediately after sacrifice. From the globes, a group of lenses was dissected within 2 h post-mortem and cultured in EMEM (containing 1.8 mM \( \text{Ca}^{2+} \)) for a 48 h incubation settling period as described in Chapter 3.

**Preparation of treatment solutions**
SJA6017 was synthesised by the University of Canterbury Chemistry Department and solubilised in DMSO. 1mg/mL ionomycin (\( \text{Ca}^{2+} \) ionophore, Sigma) was prepared in DMSO. The final concentration of DMSO in the culture media, after the addition of the inhibitor and ionomycin, was less than 0.05%.

**Experimental protocol**
After the 48 h pre-settling period in EMEM, intact lenses were divided into three groups for the experiment; control group (Control), ionomycin-induced group (Iono) and ionomycin treated with SJA6017 group (I+SJA). A group of 6 lenses was cultured under normal conditions in EMEM (Control). For inhibitor treatment (I+SJA), a group of 6 lenses was pre-treated with 100 \( \mu \text{M} \) of SJA6017 for 24 h prior to exposure to ionomycin.

At the start of the experimental period \((t = 0)\), 5 \( \mu \text{M} \) of ionomycin was introduced to the two groups (Iono and I+SJA) to induce opacity. A complete change of culture media with the
appropriate compounds was made after the first 48 h of the experimental period (t = 48 h). All lenses were monitored over the total 96 h experimental period (t = 96 h).

**Grading of opacification**

Lens opacity was scored and a grade allocated at 48 h and 96 h in the experimental period by image analysis system described in Chapter 3 (3.2).

**Analysis of calpain activities and protein profiles**

At the end of the 96 h experimental period, all lenses were harvested and the whole lenses were homogenised as described in Experiment 3 (4.5.1), Chapter 4. Soluble proteins were used for calpain activity determination using casein zymography. Urea soluble proteins were extracted and used for protein profiles by SDS-PAGE as described in Chapter 4 (4.2.1)

### 5.2.1.2. Results

**Lens Transparency**

Lens transparency was monitored over a 96 h experimental period (Figure 5.1). The degree of lens opacification was digitally analysed to confirm the visual assessment. Normal lenses cultured in EMEM remained transparent throughout the experiment.

During the first 48 h experimental period, the lenses in ionomycin (Iono) developed full opacity except for some partial clearance in the centre of the anterior region. The SJA6017 treated lenses (I+SJA) were significantly less opaque (P < 0.05). Full opacification was restricted to the equatorial region of these lenses.

All treated lenses (Iono and I+SJA) showed greater opacification by the end of the 96 h experimental period. While ionomycin-induced lenses without inhibitor (Iono) showed dense opacity, the anterior part of lenses treated with SJA6017 (Iono+SJA) still remained semi-transparent (P = 0.055).
Figure 5.1 Digital images of lenses (n= 6) taken at three different time (t= 0, 48 h and 96 h) during culturing in EMEM + 5 μM ionomycin (Iono), EMEM + 5 μM ionomycin + 100 μM of SJA6017 (I+SJA). Normal lenses cultured in EMEM are also displayed at the end of 96 h experimental period (96-C). Mean opacification score (± sd) for six lenses of each group is displayed below each image. Score of 1 represents a transparent lens. Score of 100 represents a full opacity lens.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No of lenses</th>
<th>Lens wet weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>*1.05 ± 0.05</td>
</tr>
<tr>
<td>Ionomycin (Iono)</td>
<td>5</td>
<td>1.37 ± 0.30</td>
</tr>
<tr>
<td>Ionomycin + SJA6017 (I+SJA)</td>
<td>6</td>
<td>1.43 ± 0.24</td>
</tr>
</tbody>
</table>

Two sample t-test; *P < 0.05 relative to I and Iono+SJA.

Proteolysis of lens proteins

At the end of the 96 h experimental period, calpain II activity of whole lenses was assayed with casein zymograms (Figure 5.2), although the opacities were mainly in the cortical region.
of the lens. Intact calpain II activities of both SJA6017 treated and SJA6017 non-treated lenses in the presence of ionomycin were lower than the intact calpain II activities in the control lenses.

The urea soluble protein profiles of lenses by SDS-PAGE, however, indicated partial protection by SJA6017 of spectrin in the ionomycin-induced lenses incubated with the inhibitor (Figure 5.2, (B)). In contrast, ionomycin treated lenses contained no intact spectrin in the urea soluble proteins. There was some evidence of protection of the lens specific intermediate filament, filensin (115 kDa) by SJA6017 in the ionomycin treated group with the inhibitor, compared to ionomycin treatment lenses without the inhibitor.

Figure 5.2 (A) Casein Zymography of calpains present in 100 μg of soluble protein from ovine lens after culture in three treatments for 96 h. Purified calpain II, extracted from ovine lung, was loaded in the first lane (L). Lanes 2,3 and 4 contained soluble proteins from a control lens (C), a lens treated with 5 μM ionomycin (Iono) and a lens treated with 5 μM ionomycin with 100 μM SJA6017 (I+SJA) respectively. Solid and broken arrows indicate intact calpain II and autolysed calpain II respectively. (B) SDS-PAGE of (15 μg) urea soluble protein in ovine lens at the end of the 96 h experimental period. The molecular marker was loaded in the first lane (Mm, Precision Plus Protein Standards™, All Blue, Bio-Rad). Lanes 2,3 and 4 contained urea soluble proteins from a control lens (C), a lens treated with 5 μM ionomycin (Iono) and a lens treated with 5 μM ionomycin with 100 μM SJA6017 (I+SJA) respectively. Black arrows indicate intact spectrin (280 kDa) and intact filensin (115 kDa)
5.2.2. Experiment 1-b: Calpain inhibitor in the hyper-Ca\textsuperscript{2+} medium model

5.2.1.2. Methods

Lens culture
Pairs of lenses were cultured in EMEM (containing 1.8 mM Ca\textsuperscript{2+}) for a 48 h incubation settling period according to the culture protocol described in Chapter 3.

Preparation of treatment solutions
2 M Ca\textsuperscript{2+} was made in sterilised dH\textsubscript{2}O, and added to 10 mL culture medium to make a final concentration of 5 mM Ca\textsuperscript{2+} per lens. SJA6017 in DMSO was prepared as described in 5.2.1.1. The final concentration of DMSO in the culture medium, after addition of the inhibitor, was less than 0.05%. Same volume of DMSO (less than 0.05% in EMEM) was added to the media containing 5 mM Ca\textsuperscript{2+} so there were no effects attributed to DMSO.

Experimental protocol
Three pairs of lenses were used for inhibitor treatment. One lens from each pair was pre-incubated with 0.8 \mu M SJA6017 for 2.5 h whilst the other lens from the same pair remained in EMEM. Then 16 \mu L of 2 M Ca\textsuperscript{2+} solution was added to all three pairs of lenses. The final concentration of Ca\textsuperscript{2+} per lens was 5 mM Ca\textsuperscript{2+}. All the treated lenses were cultured for 24 h. At the end of the 24 h treatment period, digital images of the lenses were taken, and all lenses were immediately harvested and cleaned with dH\textsubscript{2}O. The wet weight of the individual lens was recorded, prior to being stored at -80\degree C.

Analysis of calpain activity and protein profiles
Lenses were thawed and dissected into cortical (outer fiber cells including lens capsule and epithelium, approximately 30% of total wet weight) and nuclear regions (core region of fiber cells, 70% of total wet weight). These samples were immediately homogenised and soluble proteins and urea soluble proteins were obtained as described in Experiment 3 (4.5.1) and Experiment 1 (4.2.1) in Chapter 4 respectively. Soluble proteins were used to run casein zymography as described in Experiment 1 (4.2.1), Chapter 4. Urea soluble proteins were used to run SDS PAGE, Western detection for \(\alpha\)-spectrin (\(\alpha\)-fodrin, Affiniti) and 2-DE for proteolysis analysis as described in Experiment 1 (4.2.1), Chapter 4.

87
5.2.2.2. Results

**Lens transparency**

During the 24 h incubation period, the lenses in 5 mM Ca\(^{2+}\) containing medium showed even light scattering in the superficial region of the lens (Figure 5.3). The rest of the lens remained transparent. There were no visual signs of lens hydration.

The light scattering was completely prevented in the lens from the same pair cultured in the presence of SJA6017 in the hyper-Ca\(^{2+}\) medium. All three pairs of treated lenses showed consistent results. The calpain inhibitor, SJA6017, significantly reduced the development of lens opacification induced by the high Ca\(^{2+}\) containing medium (P < 0.05).

![Control](image1) ![SJA6017 + Ca\(^{2+}\)](image2) ![Ca\(^{2+}\)](image3)

Figure 5.3 Digital images of ovine lenses on a grid at the end of 24 h experimental period: a lens in EMEM (Control) and a pair of ovine lenses exposed to 5 mM Ca\(^{2+}\) containing 0.8 μM SJA6017 (SJA6017 + Ca\(^{2+}\)) and 5 mM Ca\(^{2+}\) only (Ca\(^{2+}\)). Mean opacification score (± sd) of each treatment is displayed correspondingly. Score of 1 represents clear transparency of lens, while 100 represents full opacity of lens. *Paired t-test (P < 0.05) of Ca\(^{2+}\) group of 3 lenses relative to SJA6017 + Ca\(^{2+}\).

**Protein content in cortex region of lens**

The wet weights of the high Ca\(^{2+}\) treated lens pairs (1.016 ± 0.021 g) were not significantly different to the wet weights of normal lenses (1.039 ± 0.083 g) at the end of 24 h treatment period. The total soluble protein contents of the cortical fiber cells were not significantly different between lenses with and without SJA6017 under the hyper Ca\(^{2+}\) condition after a 24 h experimental period. The analysis of urea soluble proteins from the pellet of the cortex showed, however, that under high Ca\(^{2+}\) there was less urea soluble protein in lenses treated with SJA6017 than in the lenses without SJA6017 (*P < 0.05, Table 5.2).
Table 5.2 Mean soluble protein and urea soluble proteins concentration (mg/g, ±sd) from the cortex region of the lenses after culture for 24 h in three different treatments. A pair of ovine lenses were cultured for 24 h in EMEM + 5 mM Ca\(^{2+}\) (Ca\(^{2+}\)) and EMEM + 5 mM Ca\(^{2+}\) + 0.8 µM SJA6017 (Ca\(^{2+}\) with SJA6017).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Soluble protein</th>
<th>Urea soluble protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+}) with SJA6017</td>
<td>3</td>
<td>(441.7 ± 22.4)</td>
<td>(2.6 ± 0.9)</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>3</td>
<td>(423.5 ± 53.9)</td>
<td>*(3.8 ± 0.6)</td>
</tr>
</tbody>
</table>

*Paired t-test of urea soluble protein in Ca\(^{2+}\) with SJA6017 group to Ca\(^{2+}\) group (P < 0.05).

**SDS-PAGE and Western detection**

Intact spectrin in urea soluble protein fractions was closely examined by SDS-PAGE (Figure 5.4, A) and western analysis (Figure 5.4, B). According to the SDS-PAGE technique, lenses treated with high Ca\(^{2+}\) contained less intact spectrin, as the intensity of the intact spectrin band was weaker than that found in the control and ionomycin treated lenses. This results provide evidence that proteolysis occurred in the lenses incubated in a hyper-Ca\(^{2+}\) medium.

![Figure 5.4](image)

**Figure 5.4** Urea soluble protein profiles of ovine lenses after culture for 24 h in EMEM (C), EMEM + 0.8 µMSJA6017 + 5 mM Ca\(^{2+}\) (S), and EMEM + 5 mM Ca\(^{2+}\) (Ca\(^{2+}\)). (A) SDS-PAGE analysis of 10 µg urea soluble proteins extracted from cortex fiber cells. Mm is a molecular marker (kDa) with Streptactin (from Bio-Rad). (B) Western detection of α-spectrin (α-fodrin, Affiniti) with 15 µg urea soluble protein from cortical fiber cells. Solid arrows indicate intact spectrin (280 kDa) and broken arrows indicate breakdown products of α-fodrin spectrin.

Evidence for proteolysis was confirmed by Western analysis of α-spectrin. The intensity of the intact spectrin band of the lenses treated in high Ca\(^{2+}\) was less than the control and SJA6017 treated lenses. Lenses treated with SJA6017 in the presence of ionomycin showed there were no changes in the intensity of spectrin using both SDS-PAGE and western α-spectrin analysis. Three major fragments of intact α-spectrin appeared in the urea soluble...
fractions in all the three treatment groups (indicated by broken arrows in (B), Figure 5.4). The intensity of the bands in all treatment groups was similar.

*Two-dimensional electrophoresis (2-DE)*

Soluble proteins were extracted from the cortex region of all treatment lenses to examine the profiles of the lens soluble protein, crystallin. Data showed there was a reduction in intact βA3- and βA4-crystallins from the lens treated with 5 mM Ca²⁺ compared to lenses incubated with EMEM (control) or 5 mM Ca²⁺ with SJA6017. All lenses contained phosphorylated αA-crystallin in the soluble fractions of the cortical region. There was, however, a high level of modified phosphorylated αA-crystallin in the high Ca²⁺ treated lenses compared to the control lenses or the calpain inhibitor treated lenses.

![2-DE protein profile](image)

*Figure 5.5* 2-DE protein profile of 50 µg soluble protein extracted from cortex region of ovine lenses after culture in EMEM (A), EMEM + 5 mM Ca²⁺ (B) and EMEM + 5 mM Ca²⁺ + 0.8 µM SJA6017 (C), for 24 h. Intact αA-ph (phosphorylated αA), βA3 and βA4 crystallin are marked by solid arrows (A). Mm are molecular markers (Precision Plus Protein Standards™, All Blue, Bio-Rad). A broken arrow in (B) indicates modified phosphorylated αA-crystallin.
5.2.3. Discussion

Application of SJA6017 to the OLCO models

SJA6017 partially protected ovine lenses from opacification. The centres of the lenses treated with SJA6017 was clearer than the lenses without SJA6017 treatment. Thus the presence of this calpain inhibitor slowed the progress of cortical opacification. Partial prevention of the loss of transparency coincided with the partial protection of intact spectrin from degradation. A fiber specific intermediate filament, filensin, also remained intact in the presence of SJA6017 with ionomycin, and was protected from partial degradation (Figure 5.2). Some cytoskeletal proteins are prime substrates for calpain degradation, and are excellent biochemical markers for calpain activation (Shoeman & Traub, 1990; Truscott et al., 1990). In rat lens models, degradation of these cytoskeletal elements has been prevented by SJA6017 (Fukiage et al., 1997b) and calpain inhibitors (Azuma et al., 1992; Sanderson et al., 1996).

E64 and SJA6017 have been effective in reducing Ca\(^{2+}\) ionophore-induced nuclear cataract formation in cultured young rat lenses (age between 5 days-4 weeks old) (Azuma et al., 1992; Fukiage et al., 1997b; Iwasaki et al., 1995; Nakamura et al., 2003). In contrast, E64 failed to prevent cortical opacity in adult rat lenses (Iwasaki et al., 1995), and calpeptin and MDL28170 (Sanderson et al., 1996) were able to partially protect against developing cortical opacity induced by Ca\(^{2+}\) ionophores in adult rat lenses (age between 10-12 weeks). These studies concluded that cortical opacification may not be due to proteolysis by calpain since inhibition of cortical β-crystallin proteolysis by the calpain inhibitor, E64, did not reduce opacification (Iwasaki et al., 1995). This study suggested that cortical opacification was due to water and electrolyte uptake. On the other hand, the study by Sanderson et al. (1996) demonstrated that calpain activation is involved in experimental cortical cataracts in mature rat lenses. These researchers argued that the partial protection of lens transparency was explained by ionomycin-induced opacity condition, where the cortex region of lens faces osmotic stress after initial disruption of the submembrane cytoskeleton by calpain-induced proteolysis. Subsequently, complete protection of cytoskeletal elements is impossible under the sustained Ca\(^{2+}\) increase in the lens.

In a whole lens system, lens permeability to calpain inhibitors must be considered when investigating the role of calpains in cataractogenesis. The ability of a molecule to penetrate
cells is a factor determining the apparent potency of the inhibitor (Burstein, 1985). Thus, despite SJA6017 having a low IC\textsubscript{50} value (approximately 80 nM, personal communication with Janna Nicole, The University of Canterbury) in an \textit{in vitro} assay, the penetration of SJA6017 into lenses may limit its effectiveness. Consequently, there is only partial protection against changes in lens transparency. In contrast, E64d, one of the E64 derivatives, showed improved penetration to the lens and improved protection against Ca\textsuperscript{2+} ionophore-induced nuclear opacity, even though it had weaker inhibition of calpain II activity than E64 in an \textit{in vitro} assay (Azuma \textit{et al.}, 1992). In addition, when the IC\textsubscript{50} of calpain inhibitors was determined in a cell-free system with rat lenses, it was also found that the IC\textsubscript{50} values of calpain inhibitors were substrate dependent and in sub-micromolar ranges (Sanderson \textit{et al.}, 1996).

Increased calpain activation occurred in both the ionomycin-treated and the ionomycin plus SJA6017-treated lenses based on the casein zymography. There were no differences in calpain activation levels between the lenses with and without the SJA6017 treatment. This could be explained as there was nearly full opacity in both treated lenses by the end of the 96 h experimental period. The analysis of whole lenses, instead of the opaque cortex regions, probably reduced any differences in localised calpain activities.

Ovine lenses in the OLCO model were exposed to a continuous influx of Ca\textsuperscript{2+} by the ionomycin treatment. The continuous influx of Ca\textsuperscript{2+} resulted in lens opacity and cell death (see Chapter 4). In Chapter 4, it was proposed that cytoskeletal degradation by calpain-induced proteolysis is the cause of the opacification. It is speculated that the protection of intact cytoskeletal elements by the calpain inhibitor may only be effective in the early stage of opacification, but would not be sufficient enough to sustain cell membrane integrity under the constant influx of Ca\textsuperscript{2+} in the OLCO model. The present calpain inhibitor study confirms that the cortical opacification is a result of the complex pathological conditions under the chronically high Ca\textsuperscript{2+} environment, which is followed by cross-linked aggregation, uptake of water and electrolytes, and cortical cell death (Chapter 4).

\textit{Application of SJA6017 to the hyper-Ca\textsuperscript{2+} medium opacification}

The nature of the opacification induced by hyper-Ca\textsuperscript{2+} medium is different to the OLCO model. For example, the hyper-Ca\textsuperscript{2+} medium results in the opacification on the superficial
area of the lens and there is no sign of lens hydration. This sub-capsular nature of the opacification has also been observed in rabbit (Hightower et al., 1985) and human (Hightower & Farnum, 1985) lenses cultured in Ca²⁺-rich medium.

In early studies on rabbit lenses, Hightower et al. (1985) demonstrated an increase in free Ca²⁺ levels after culturing lenses in HEPES buffered medium with 20 mM Ca²⁺ for 20 h at 22°C. That study showed little change in Na⁺ content and no hydration in the cultured lens, but the entire surface of the cortex was opaque at the end of the incubation period. The interesting feature of the reported studies was that subsequent culturing of these opaque lenses in culture medium (TC 199) at 37°C restored their transparency and electrochemical gradients. When human lenses were exposed to the same Ca²⁺-rich medium under the same culture condition as the rabbit lenses (Hightower & Farnum, 1985), the human lens developed localised opacities often observed in human cataracts. The opacity was confined to the outer, superficial regions of the lens cortex and limited to the sub-capsular regions. The process in the opaque human lens was, however, irreversible.

In the current research with ovine lenses, the complete protection by the calpain inhibitor, SJA6017 of the development of opacification (Figure 5.3) supports the involvement of Ca²⁺-induced proteolysis in the opacification process. A recent study with porcine lenses showed that applying the calpain inhibitor, SJA6017, protected the lens from hyper-Ca²⁺ medium-induced opacification (Biswas et al., 2004). The presence of SJA6017 had no significant effect upon Ca²⁺ uptake by porcine lenses but slowed the early stages of opacification. The ability of SJA6017 to retard cataractogenesis in porcine lenses may be due to its ability to inhibit calpains, which have been activated by the elevated levels of lens Ca²⁺.

The results in this thesis also demonstrate that the protection of lens transparency is accompanied with a reduction in insoluble lens proteins (Table 5.2) and lens protein degradation (Figure 5.4 and 5.5). Previous research has reported that high Ca²⁺ levels decrease the chaperone capacity of α-crystallins by altering their structural integrity, and increasing the susceptibility of other crystallins to proteolytic degradation (del Valle et al., 2002). The addition of SJA6017 to the culture media reduced the partial degradation of intact βA3 and βA4, and there was no sign of degradation of phosphorylated αA-crystallins. In this
respect, opacification induced by the hyper-Ca\(^{2+}\) medium is the result of Ca\(^{2+}\)-activated proteolysis, which is prevented by calpain inhibitors.

Despite the reduction of crystallin degradation, treatment with SJA6017 did not change the total amount of soluble proteins compared to the lenses treated in the hyper-Ca\(^{2+}\) medium without SJA6017. This observation is similar to the results with the OLCO model (Chapter 4), where broad opaque areas were displayed in the lenses and there was no evidence of changes in the soluble protein fractions.

Although there was a reduction of intact spectrin in the urea soluble fraction from the hyper-Ca\(^{2+}\) treated lenses (Figure 5.4.), the profiles of the \(\alpha\)-spectrin fragments were similar to those from the control lenses (cultured with no treatment) and lenses treated with Ca\(^{2+}\) plus SJA6017. Previous research has shown that insoluble proteins from fresh (non-cultured) rat lens exhibited 145 and 150 kDa molecular weight fragments along with intact \(\alpha\)-spectrin (Fukiage et al., 1997b). There was no change in their concentration after the lenses were cultured in normal medium for 5 days. Proteolysis of \(\alpha\)-spectrin to discrete fragments has been associated with changes in cell shape and membrane morphology, which occur in many cell types (Morrow et al., 1997). In the lens, the cleavage of \(\alpha\)-spectrin to 145 and 150 kDa fragments has also been observed during terminal differentiation and in ageing fiber cells (Lee et al., 2001). It is possible that the appearance of 145 and 150 kDa fragments in the cortical region of the normal ovine lenses is a part of lens differentiation. It has also been reported that \(\alpha\)-spectrin fragments are more abundant in supernatant fractions than pellet fractions (Lee et al., 2001). It is apparent that the degradation of spectrin must be investigated in the total proteins of the lens, soluble (supernatant) and insoluble (pellet + urea soluble) fractions.

The lens has limited extracellular spaces. This limits penetration of compounds into the lens and potentially reduces their effectiveness. In rats, the lens epithelium and cortex is where most of the calpain II activity resides (Ma et al., 1998). Studies have reported that SJA6017 (IC\(_{50}\) = 80 nM) is a potent calpain inhibitor compared to E64 (2.31 \(\mu\)M) or leupeptin (0.49 \(\mu\)M) (Fukiage et al., 1997b). Effective inhibition of spectrin from proteolytic degradation was achieved by low concentration of calpain inhibitor in blood cells (Mehdi et al., 1988), where the cell structure is different to the lens. In the present research, the total protection by SJA6017 against lens opacification induced by the hyper-Ca\(^{2+}\) culture medium could be due
to the limited penetration to the surface opaque site of the lens by the calpain inhibitor with a low IC₅₀ value. Thus, the efficacy of calpain inhibitors as retarders of cataractogenesis probably depends on the permeability of the drug and the nature of the opacification to which it is applied.

Overall, the two Ca²⁺-induced cataractogenesis models with the calpain inhibitor, SJA6017, demonstrated that calpain was involved in the development of opacity in ovine lens. The data suggest that the hyper- Ca²⁺ medium induced opacification lens culture system is a useful assessment system for testing the efficacy of novel calpain inhibitors as potential candidates for retarding the progression of cataract in in vivo lenses.

5.3. Experiment 2  Assessment of novel inhibitors in vitro and in vivo

A study on the structural activity relationships of SJA6017 and its analogues revealed that for potent inhibition of calpain, an aromatic group at the P₃ position and a bulky amino acid residual aldehyde at P₁ is favoured (Inoue et al., 2003) (Figure 5.6). Calpains were found to prefer leucine or valine in the P₂ position of small peptide substrates. The reactive aldehyde forms a hemithioacetal with the active site cysteine thiol in a Ca²⁺ dependent manner (Sasaki et al., 1984). P₃ is a N-terminal protecting group (Inoue et al., 2003). Therefore the potential exists for the synthetic modification of small peptide aldehyde compounds such as SJA6017 to produce novel structures with calpain inhibitory activity.

![Figure 5.6 The structure of dipeptide calpain inhibitor, SJA6017 (N-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal). P₁, P₂ and P₃ in grey areas are positions counted from the point of cleavage, and represent positions of L-leucine, L-valine and N-4-Fluorophenylsulfonyl respectively.](image-url)
Two dipeptide-based compounds, labelled as Cat0059 and 4583, were developed and synthesised by the University of Canterbury-Chemistry department. Primary structures of the two novel compounds were related to the structure of SJ6017, where P1, P2 and P3 positions were modified to improve solubility characteristics and inhibitory effect.

Initially, in vitro assays were conducted by the University of Canterbury Chemistry Department. Using BODIPY-fluorescent labelled casein as a substrate for calpain II (BODIPY assay) (Jones et al., 1997; Thompson et al., 2000), the two compounds displayed effective inhibitory activity to purified calpain II from ovine lungs. Consequently, they are potential novel calpain inhibitors.

5.3.1. Novel calpain inhibitors in an in vitro culture system

In this experiment, Cat0059 and 4583 were assessed for their efficacy as calpain inhibitors in the in vitro culture system, with the subsequent objective of applying the inhibitor to retard the progression of cataract in the in vivo cataract inherited ovine model.

5.3.1.1. Methods

Lens culture
Globes were obtained from sheep aged younger than one year old. Pairs of lenses were dissected from the globes and immediately placed in the culture system (using EMEM) as described in Chapter 3.

Preparation of treatment solution
2 M Ca^{2+} in sterilised dH_{2}O was added to 10 mL culture medium to make a final concentration of 5 mM Ca^{2+}. Two calpain inhibitors (Cat0059 and 4583) were also solubilized in DMSO. The final concentration of DMSO in EMEM, after the addition of the inhibitor, was less than 0.05%. Adequate amounts of DMSO was added to the medium containing 5 mM Ca^{2+} so there was no effect attributed by DMSO.
**Experimental protocol**

After a 48 h pre-incubation period, 3 pairs of lenses were used to assess the inhibitory effect of each of the two novel calpain inhibitors at 0.8 μM concentration, against the development of the opacification induced by 5 mM Ca\(^{2+}\). One of each of the paired lenses, was pre-incubated with one of the inhibitors in EMEM for 2.5 h, whilst the other was cultured in EMEM. Both lenses were subsequently exposed to a final concentration of 5 mM Ca\(^{2+}\) in EMEM, by the addition of 16 μL of 2 M Ca\(^{2+}\) stock solution in dH\(_2\)O. All lenses were incubated at 37°C under 0.5% CO\(_2\), for either 20 h (Cat0059) or 24 h (4583) experimental periods.

**Visual image analysis**

All lenses were monitored at the end of the experimental period. Lens opacity grade was scored by an image analysis system that was programmed to grade the extent of opacification captured by a digital photo image (refer to Chapter 3, 3.2).

5.3.1.2. Results

Two novel inhibitors from first generation (Cat0059) and second generation (4583) modification of SJA6017 were assessed and compared for their ability to prevent opacification due to exposure to 5 mM Ca\(^{2+}\) culture medium solution. The loss of transparency was significantly prevented by Cat0059 (P < 0.01) and 4583 (P < 0.005) in a paired t-test (Figure 5.7).

![Figure 5.7](image)

**Figure 5.7**. Digital image of ovine lenses and their mean opacification grading scores (± sd) on paired ovine lenses after culture in EMEM + 5 mM Ca\(^{2+}\) (Ca\(^{2+}\)) and EMEM +5 mM Ca\(^{2+}\)+0.8 μM inhibitor (Ca\(^{2+}\) with 4583 or Ca\(^{2+}\) with Cat0059) for 24 h (4583) or 20 h (Cat0059) experimental periods. Number of pairs used for each inhibitor were three (n = 3); Opacification scores represent 100 = Full opacity, 1 = clear and transparent. Paired t-test; *P < 0.005 and **P < 0.01 relative to Ca\(^{2+}\) only treated eye.
5.3.2. Topical application of novel calpain inhibitors to in vivo cataract model

The purpose of the experiment was to assess the inhibitory effect of topical administration of calpain inhibitor molecules (Cat0059 and 4583) on the progress of cataract formation in the inherited in vivo ovine model over 11 weeks.

5.3.2.1 Methods

Ethics approvals
Lincoln University Animal Ethics Protocol-ACE#60.

Preparation of two eye-drops
A standard eye-drop formula containing 0.1% w/v (or 1 mg/mL) inhibitor was prepared for Cat0059 and 4583 inhibitors. Each inhibitor was dissolved with 14% ethanol to make final 3 mM concentration (Cat0059) or 2 mM (4583) in a standard eye-drop solution (RD5721).

Sterile eye-dropper bottles were used to hold approximately 5 mL of the final eye-drops formula per bottle, and stored at 4°C. The volume of each eye drop was approximately 40 µL. Topical drug formulation must be tolerated, chemically and microbiologically stable, and readily release the active ingredient (Lee & Robinson, 1986; Piercy, 1985). A standard eye-drop solution (RD5721), therefore, contains; 0.9% sodium chloride (physicochemical parameters similar to those of tears to reduce irritation), 0.3% hydroxypropyl methyl cellulose (HPMC-RG-10T) (to increase viscosity of the eye drops so to increase the residence time of the formulation in the precorneal space), 0.05% disodium EDTA (for microbiologic preservation), 0.01% benzalkonium chloride (stabiliser) and dH₂O.

Pre-treatment test for eye irritation with Cat0059 and 4583 eye-drops
Each eye drop was tested on a single non-cataract lamb (aged 2.5 months). A single eye-drop was applied topically to the right eye of the lambs, and the eye inspected after 3 h. Then eye drops were then applied every half an hour for the next 5 h. The lamb's eyes were monitored closely by Dr Rob Macfalane (Veterinary) for signs of any irritation.
Classification (scoring) for in vivo ovine cataract

Lamb eyes were inspected in a darkened room for cataract formation and severity by Veterinary Ophthalmologist, Dr Steve Heap. Eye pupils were dilated with atropine (1% atropine sulphate, Sigma Pharmaceuticals, Australia) and the lenses inspected using a slit lamp (Kowa, SL/5, Japan) and ophthalmoscope (Vista Diagnostic Instruments, Kellar, UK). Slit lamp and ophthalmoscope viewing of a lens is the typical clinical examination performed on human patients to allow diagnosis of cataract formation. The standard cataract scoring that had been previously developed (Robertson et al., 2005) was used in this trial (Figure 5.8).

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No opacity seen in the lens (normal lens)</td>
</tr>
<tr>
<td>1</td>
<td>Small opacity detected at either the anterior or posterior suture lines</td>
</tr>
<tr>
<td>2</td>
<td>Small opacities detected at both suture lines</td>
</tr>
<tr>
<td>3</td>
<td>Opacification at sutures and mild cortical involvement</td>
</tr>
<tr>
<td>4</td>
<td>Moderate to severe cortical development</td>
</tr>
<tr>
<td>5</td>
<td>Immature cataract involving whole lens</td>
</tr>
<tr>
<td>6</td>
<td>Mature cataract</td>
</tr>
</tbody>
</table>

Figure 5.8 Progression of the ovine inherited cataract and cataract scoring system developed for the inherited ovine cataract using ophthalmoscopy and slit lamp microscopy (adapted from Robertson et al., 2005)

Selection of lambs for inhibitor trial treatments

A number of lambs were bred from three cataract sires (I.D. of 37/02, 38/03 and 102/03) and a flock of normal-eyed unrelated Coopworth ewes. Lambs born around August and September 2004, were housed in grass paddocks containing perennial rye grass and white clover at Lincoln University, New Zealand. Eyes of all new lambs were assessed for cataract progression and their cataract scorings were recorded on the 16th November 2005 by Dr Steve Heap. Sixty five had cataracts and fifty eight had normal eyes.
Forty young lambs (2-3 months old) with cataract scores between 1 and 3 were selected. Ten control lambs without cataract were also selected. There were two eye-drop treatment groups (one group treated with an eye-drop containing Cat0059 and the other treated with an eye-drop containing 4583). Each eye-drop treatment group consisted of 20 cataract lambs and 5 normal lambs. Each group of lambs had a similar proportion of eyes at various stages of cataract with scores between 1 to 3, a similar ratio of sex (ram:ewe of 14:11) and body weight distribution (25.7 ± 4.5 kg and 25.9 ± 6.3 kg). Each eye-drop treatment group of 25 lambs was marked with different colour paint on the head for identification during eye-drop administration. Otherwise, the two treatment groups were run as one flock.

Protocol for eye-drop treatments
From 1st December 2004 through to mid-February 2005 (total 11 weeks trial), the flock was yarded three times daily. The right eyes of all lambs were treated with a single eye-drop, while the left eye had no eye-drop applied as an untreated control. The treatment took less than 30 min for both treatment groups. Each treatment group received eye-drops together at one time, to prevent any handling errors.

During the 11 week eye-drop experimental trial period, the cataract progression of 50 lambs was assessed three times by Dr Steve Heap at 10, 37 and 67 days after the beginning of the treatment. Lenses were visualised through atropine-dilated pupils using a slit lamp and an ophthalmoscope and scored on a 0-6 point scale (Figure 5.8).

Sample collections
For each eye-drop treatment group, twelve lambs (2 non-cataract and 10 cataract lambs) were randomly selected and sacrificed within 12 h after the last eye-drop application at the end of 11 week trial period. The eye globes were then removed.

Approximately 500 μL of aqueous humour was collected by corneal puncture with an insulin syringe and stored in Eppendorf tubes. Each cornea was dissected and rinsed with dH2O several times to remove any remaining eye-drop residues. Lenses were dissected from globes using a posterior approach and placed in separate labelled tubes. Small portions of liver and muscle tissue from 5 lambs per treatment were collected to test for inhibitors in other parts of the animal. All cornea, aqueous humour, lens, muscle and liver samples were weighed and frozen at -80°C.
Extraction of calpain inhibitors from dissected samples

**Homogenisation of samples:** The corneas was cut from eye globes and ground into powder using a hand mortar and pestle under 3-5 mL of liquid nitrogen, and then transferred into a tube with 5 mL of dH₂O. 400 μL of aqueous humour per lens was mixed with 1 mL of dH₂O prior to addition of 10 mL of ethyl acetate (CH₃COOC₂H₅). The cortical region of each lens was dissected and homogenised in 1 mL of dH₂O with a sonicator (Ultrasonic Processor, Heat Systems-Ultra Sonsics, INC, N.Y.). Neck muscle samples (9 g) and a sample of liver (13 g) were homogenised with 5 mL of dH₂O using a Polytron PT 3100 homogeniser with a Polytron PT-DA 3012/ 2T mm aggregate tip for 20 s at 25,000 rpm speed.

**Extraction:** 10 mL of ethyl acetate was added to each homogenate and shaken by hand for approximately 30 s. The upper phase was collected into a labelled 50 mL tube and the water phase was washed with 10 mL ethyl acetate and shaken as before. The upper phase was added into the same 50 mL tube. Each extracted sample in a total volume of 20 mL was concentrated to 1.5 mL and filtered through a 0.2 μm PTFF membrane into a 2 mL Eppendorf tube. The filtrate was evaporated to dryness in a SpeedVac Concentrator (SAVANT Instruments INC, N. Y.). Samples were then resuspended in 100 μL of ethyl acetate for HPLC analysis.

**Determination of UV spectrum of inhibitors**
1 mg/mL of calpain inhibitor (Cat0059 or 4583) were solubilized in 50:50 acetonitrile and dH₂O solution. 2, 4, 6 or 10 μL of the inhibitor solution was mixed with 1 mL dH₂O for spectrometer reading. These samples were then scanned between 190 nm and 450 nm spectrum with a UV/Vis spectrometer (UNICAM, UV-400, England) to identify absorbance peaks for the inhibitors.

**Measurement of extracted samples by HPLC (High Performance Liquid Chromatography)**
A series of standard solution of known concentration (0, 2, 10, 25, 50 and 100 ng/μL) of calpain inhibitor (Cat0059 or 4583) were solubilized in 50% HPLC grade acetonitrile (CH₃CN) and 50% dH₂O (unless otherwise stated) solution at room temperature. The concentrated sample was filtered through a PTFF membrane with 0.2 μm pore size (BioLab). Calibration curves were prepared by injecting the series of different concentrations in 5 μL of standard, including a solvent blank run (50:50 of acetonitrile:dH₂O). 5 μL of each filtered sample was analysed immediately after the standard solutions.
All injection samples were separated by a Proteo (C12) Reverse Phase Column (Jupiter™ 4µ Proteo 90Å, size: 250 x 4.6 mm, with pore size 90Å, Phenomenex, Part No. 00G-4396-E0), at 40°C. Flow rate was maintained at 1 mL/min. Two mobile phase solvents used for separation were filtered 0.2% Trifluoroacetic acid (TFA, C₂HFO₂) in dH₂O (pH = 1.8) and HPLC grade acetonitrile. Absorbance (AU) of standard (y-axis) was plotted against known amount (ng) (x-axis).

5.3.2.2. Results

*Pre-treatment test for eye irritation with two inhibitor eye-drops*

After 8 hours, no irritation was observed in the treated eye.

*Observation of cataract progression in lambs treated with inhibitors*

The cataract scores for two eye-drop treatment groups were taken two weeks before the start of the trial (t = -14) and three times (t = 10, 37, 67 days) during the total 77 days (11 weeks) treatment period. The mean scores of each reading for each eye-drop treatment group are illustrated in Figure 5.9. Cataract scores for the Cat0059-treated eyes were the same as the untreated eyes over the 37 day treatment period. At the 67 day treatment period, the eyes treated with Cat0059 showed a higher cataract score than the untreated eyes (P < 0.05). Throughout the 67 day eye-drops application period, the cataract scores from 4583-treated eyes were not significantly different from the untreated eyes.

![Figure 5.9](image-url)  
*Figure 5.9. A plot of mean scores for two inhibitors untreated eyes and treated eyes with calpain inhibitor (Cat0059 (A) or 4583 (B)) during 67 days. Different italicised letters within examined day indicates significant difference (Paired T-test, P< 0.05). n = 20 lamb per inhibitor.*
Observation of untreated eyes over the 67 day period

The rate of cataract formation was analysed using the mean cataract scores of the untreated eyes of cataract lambs over the 67 day period (Figure 5.10).

Under the current cataract scoring system (Figure 5.8), the cataract progression of untreated lambs eyes with the initial cataract scores of 1 (stage 1) or 2 (stage 2) developed linearly over the 2 month period. The average score advanced by 1 score per month. In comparison, the progression of cataracts in the lambs with an initial cataract score of 3 (stage 3) advanced by 1.5 score in the first month, then remained with an unchanged average score in the second month.

![Progress of cataract of untreated eyes from 3 initial cataract stages (1, 2 and 3)](image)

Figure 5.10 A plot of mean cataract scores for untreated eyes over 67 days. Different letters within a group started with same initial cataract score indicates significant difference in mean cataract score between different days (Paired t-test, P < 0.05). Stage 1 (n = 14), stage 2 (n =13) and stage 3 (n = 8).

HPLC protocol for Cat0059

UV spectrum scanning of serial solutions of Cat0059 in acetonitrile revealed a strong absorbance at 300 nm, with minor ones at 190 nm and 220 nm (Figure 5.11).

With the gradient method (40°C column temperature, mobile phase solvents of acetonitrile: 0.2% TFA in dH2O), the HPLC chromatograph at 300 nm showed a sharp peak with a bell-shape (1-2 min broad) at ~18.4 min elution time (Figure 5.12). Cat0059 was eluted from the HPLC column with a gradient of 50% acetonitrile and 50% dH2O with 0.2% TFA over
a 25 min separation phase. Analysis of the UV spectrum of the peak confirmed it was Cat0059. The peak was collected and confirmed as Cat0059 by mass spectrometry. Minimum detection of Cat0059 was 3 μM (or 5 ng of Cat0059 in 5 μL HPLC column injection volume).

![UV spectrum of Cat0059](image)

**Figure 5.11** UV spectrum of 2, 4 and 6 μg/mL Cat0059. The strongest peak was absorbed at around 300 nm.

![Chromatogram of Cat0059](image)

**Figure 5.12** Chromatogram of Cat0059 (50 ng in 5 μL injection) at 300 nm UV detection with gradient methods used during development for separation and quantitation of Cat0059. The mobile phase was run as a gradient with 0.2% TFA in water. 5% (0-25 min), 100% (26-31 min) and 5% (35.5-40 min) acetonitrile (CH₃CN) (showed as a broken line).

*Detection of Cat0059 in samples*

The presence of Cat0059 was measured in cataractous lambs by HPLC. It was not detected in either the treated or non-treated eyes including the cornea, aqueous humour or cortical region. HPLC did not detect any Cat0059 in liver or muscle samples from the same lambs.
**HPLC protocol for 4583**

The UV spectrum scanning of serial solutions of 4583 in acetonitrile revealed a strong absorbance at 195 nm and with a minor one at 329 nm. With the gradient method (40°C column temperature, mobile phase solvents of acetonitrile:0.2% TFA in dH2O), the HPLC chromatograph at 329 nm showed a sharp peak with a bell-shape (1-2 min broad) at ~32 min elution time (Figure 5.14). 4583 was eluted from the HPLC column with a gradient of 75% acetonitrile and 25% dH2O with 0.2% TFA over a 40 min separation phase. Analysis of the UV spectrum of the peak confirmed it was 4583. The peak was collected and confirmed as 4583 by mass spectrometry. The minimum detection of 4583 was approximately 6.4 μM (or 15 ng of 4583 in 5 μL HPLC column injection volume).

**Figure 5.13** UV spectrum scanning results of 2, 4, 6 and 10 μg/mL 4583. There were two major absorbance at 195 nm and 329 nm by 4583 (indicated by arrows).

**Detection of 4583 in samples**

The presence of 4583 was measured by HPLC in cataractous lambs. It was not detected in the treated or non-treated eyes including the cornea, aqueous humour or cortical region. There was a trace amount of 4583 detected in the liver samples collected from 4583-treated lambs. Using the 4583 standard curve, the average concentration was 0.05 nM 4583 in the liver of treated lambs (or 22.8 ng of 4583 in 1 g wet liver sample present). HPLC of muscle samples from the same lambs did not detect 4583.
Two novel calpain inhibitors were able to decrease the opacification of lenses cultured in hyper-Ca\(^{2+}\) (5 mM) culture medium. These two inhibitors were formulated and used to treat one eye of lambs with inherited cataracts, in the form of eye-drop applications that were applied three times daily. Unfortunately, there was no evidence that either inhibitor slowed or cataract development between the treated and untreated eyes of the lambs during the 67 days treatment period. Neither inhibitor was detected in any part of the cornea, aqueous humour or the lens. These compounds therefore appear to be unable to reach the lens using the current eye-drop application treatment and procedure protocols.

In recent studies on the topical administration of SJA6017, the molecule exhibited some inhibitory effects on inherited cataracts in ovine lenses (Robertson, 2003; Robertson et al., 2005) as an eye-drop application (0.5% of SJA6017 or 15 mM SJA6017) in a pharmaceutical ophthalmic liposome preparation (supplied by Senju Pharmaceutical Co Ltd, Kobe, Japan).
This was 5 times the concentration that could be achieved in the current experiment (0.1% of the novel calpain inhibitor, or 3 mM Cat0059 / 2mM 4583). The liposome based eye-drop protocol increases the solubilisation of lipophilic molecules by emulsifying or encapsulating lipophilic molecules in liposomes (artificial microscopic vesicles consisting of an aqueous core enclosed in one or more phospholipid layers). The high drug concentration ensures there is a high gradient across the cornea and, consequently, a high diffusion rate (Havener, 1983; Lee & Robinson, 1986). In fact, only compounds dissolved in solution are able to permeate barriers (van De Waterbeemd et al., 2001). The conclusion is that compounds need to have a high solubility within an eye-drop solution.

SJA6017 has subsequently been modified to improve its physiochemical properties, such as increasing its water solubility and transcorneal permeability (Nakamura et al., 2003). Such molecules with a high trans-corneal permeability, as judged by topical administration to the eye of rabbits, significantly retarded Ca\(^{2+}\)-induced opacification in the rodent in vitro model.

The present data showed that in early stage cataracts with scores of 1 or 2, the development of cataracts progressed linearly over the trial period (Figure 5.10). Cataracts with scores of 1 or 2 are categorised by having a small opacity in the suture lines (Figure 5.8). In contrast, cataracts with a score of 3 show signs of mild cortical opacity. The pattern of the cataract progression demonstrates that the development of mature cataracts is accelerated once the cataract starts to involve the cortical regions of the lens. As observed in the culture experiments, which compared the inhibitory effect of SJA6017 between the OLCO and the hyper-Ca\(^{2+}\) medium induced models, the calpain inhibitor was less effective once the opacification had progressed beyond the initial stage.

It is therefore possible that the selection of lambs with inheritable cataract gene before cataract development could increases the chance of the calpain inhibitors being more effective at preventing cataract development, i.e. applying an inhibitor at pre-cataract stage may be the most efficient way of preventing the progress of cataract development.

Drugs can interact with proteins in ocular fluids. This can vary between individuals and species and also depend on pathological conditions. Cataractogenesis is characterised by the loss of lens proteins into the aqueous humour (Duncan et al., 1994). The protein interaction can result in the inactivation and elimination of the drug from the aqueous humour. An
apparent lack of an effect from a medical therapy viewpoint may be the result of such interactions as illustrated by the release of proteins into the aqueous humour (Mikkelson et al., 1973). This could be a contributing factor which lowers the bioavailability of the calpain inhibitors to the lens.

The calpain inhibitors, Cat0059 and 4583, were dissolved in 14 % ethanol and then formulated into an aqueous ophthalmic eye-drop solution. The use of ethanol made the eye-drop less viscous and reduced the retention time of the eye drops on the surface of the eye. It is, however, unknown whether novel calpain inhibitors are metabolised by the lens or how quickly the compound is actually cleared from the lens.

Future calpain inhibitors will require better penetration properties, higher water solubilities and lower \( IC_{50} \) level to increase their inhibitory effect through topical administration. The alternative would be a higher frequency dosing regime, which is an unrealistic treatment option to prevent cataracts in animals or humans.
Chapter 6: Drug Profile of the Novel Calpain Inhibitor, Cat0059

6.1. Introduction

For a therapeutic agent to be potent, the delivery of a drug to its target site and its bioavailability within the target site are two important factors. Topical drug delivery is the most common treatment for diseases of the anterior segment of the eye, such as glaucoma (reviewed by Schoenwald, 2003). Pharmacokinetics is the process by which a drug is absorbed, distributed, metabolized, and eliminated by the body. The relevant compartments for pharmacokinetics of ocularly applied drugs are the tear fluid, multilayered cornea, aqueous humour, lens, vitreous humour and plasma. The cornea has a lipophilic cell layer on the surface and hydrophilic structure in core layers, whilst the aqueous humour and tears are aqueous environments. As a consequence, a desirable topical drug must have both lipophilic and hydrophilic portions for optimal penetration into the eye.

The principle route of drug entry into the eye is through the cornea (Ahmed & Patton, 1985; Lee & Robinson, 1986; Piercy, 1985; Wang et al., 1991). The cornea is the transparent anterior outer covering of the eye and is continuous with the sclera. It consists of three main barriers in series: the epithelium, the stroma, and the endothelium. The corneal epithelium and endothelium are cellular layers that contain both transcellular and paracellular pathways for transport (reviewed in Havener, 1983); lipophilic molecules preferentially diffuse within cells, whereas hydrophilic molecules permeate mostly through the openings between cells. Ninety percent of the thickness of the cornea consists of the corneal stroma, which is a highly hydrated fibrous acellular tissue (reviewed in Berman, 1991). The corneal epithelium contributes the most effective barrier for drug penetration in the cornea. The corneal epithelium has annular tight cellular junctions, which completely surround and effectively seal the superficial epithelial cells. As a consequence, it is largely impenetrable to non-lipophilic agents (Boot et al., 1991; Burstein, 1985; De Kruijf et al., 1987; McDermott et al., 1990). Then the drug faces a hydrophilic corneal stroma layer to pass through to meet the second cellular layer of the cornea, the endothelium, before reaching the aqueous humour.
Interaction with proteins in ocular tissues can also markedly influence the pharmacokinetic profile of a drug, as binding to proteins can reduce the bioavailability of the drug and result in inactivation (Burstein, 1985). Aqueous humour is located in the space in front of the lens and behind the cornea (Figure 1.1 in Chapter 1). Aqueous humour has a very low protein content. Its protein content needs to be less than 1% of that found in plasma in order to maintain optical clarity and to contribute to minimum light scattering (Davson, 1990). The aqueous humour is continuously replaced with material produced through the ciliary body, which flows into the anterior chamber and then out through a drainage canal, called the Canal of Schlemm. The turnover of aqueous humour production material within the eyeball is 2.23%/min in the rat (Mermoud et al., 1996) and 2.5%/min in the mouse (Aihara et al., 2003).

As a consequence, drug elimination by the aqueous humour is a major factor limiting the penetration of the drug into the lens. The lens contains the regular arrangement of the lens fiber cells with a minimal extracellular space. The highly differentiated fiber cell structure is filled with a high concentration of soluble proteins, crystallins. This high concentration of soluble lens proteins and the cell membrane structure of the lens can significantly influence the pharmacokinetic profile of a drug.

Several models predict the absorption, distribution, and elimination of drugs in the eye. Some of these models are very complex and detailed (Friedrich et al., 1993; Grass & Lee, 1993; McLaren et al., 1993). Because of the complexity of the eye, however, the only true predictor of drug levels in the eye is experimental information. Cat0059 is a potent calpain inhibitor (IC$_{50}$ value against calpain II is 30 nM) with improved water solubility characteristics. Despite its effectiveness in preventing opacity in the in vitro studies (Chapter 5, 5.3.1.2), topical administration of the molecule in an in vivo animal model revealed there was no trace of Cat0059 in the lens using current techniques (5.3.2.2). As a part of further investigation into the drug profile of Cat0059 in a biological system, the current chapter is designed to explore the pharmacokinetics of the calpain inhibitor, Cat0059, in ocular tissues and compartments within the lens.

The studies were composed of four experiments. First, the permeability of the drug through the first barrier of the eye, the cornea, was determined by measuring the concentration of the drug in the aqueous humour after application of the drug to the cornea.
Second, the lens permeability of Cat0059 was examined using an *in vitro* culture system. The potential cytotoxicity of Cat0059 and its stability within the lens culture environment were also investigated.

Third, the binding factors of the drug in the aqueous humour and lens homogenates were evaluated. The result of this test should indicate the amount of unbound drug which is available to target compounds within the cell. Detection methods for uptake of the drug were also evaluated in this experiment.

Direct injection of a molecule into the eye globe by-passes the barriers of the eye and enhances intraocular drug concentration. Thus the technique allows a high concentration of a drug to be rapidly reached in intraocular tissues. Intravitreale injections have been used to deliver antiviral agents (Baeyens *et al.*, 1997). Water-soluble agents in the vitreous body diffuse through the gel-like material and ultimately reach the retina. There may be some leaking of the agents into the aqueous humour from where the agents can be taken up by the lens. In a final experiment, intravitreal injection of Cat0059 was used as an alternative method of delivering the drug into the aqueous humour and to the lens. This final experiment was designed to investigate the extent that other factors are operating when there is a local ocular injection of a drug.
6.2. Experiment 1: Corneal permeability of Cat0059

The purpose of the experiment was to evaluate how much Cat0059 could get through the cornea and into the aqueous humour. The trans-corneal permeability of Cat0059 was investigated by using an intact ovine globe in constant contact with an eye-drop solution containing 3 mM Cat0059 (0.1% eye-drop solution).

6.2.1 Methods

Eyeball preparation
Eye globes were removed from the eye sockets of young sheep (5 - 8 months old), immediately after sacrifice. Within 2 h they were placed in a 50 mL test tube. The diameter of the test tube was such that the eye globe fitted snugly within the walls. The anterior portion of the eye globe (cornea) was positioned upwards within the tube. Approximately 6 mL of Cat0059 eye-drop solution (3 mM Cat0059) was added to the test tube containing the eye globe, thus submerging the entire cornea. Due to the tight fit of the eye globe within the tube, solution only remained in contact with the anterior portion of the eye globe (i.e. entire surface of the cornea and small portion of sclera). Hence, the posterior portion of the eye globe did not come in contact with the Cat0059 solution during the experimental period.

Experimental protocol
A total of 7 ovine eye globes were set in individual 50 mL test tubes, and the cornea region of the eye globe faced uppermost. Five globes were submerged in eye-formula with active compound, Cat0059, for 1 h \((n = 1)\), 2 h \((n = 2)\) or 24 h \((n = 2)\) incubation periods. The remaining eye globes were left in contact with eye-formula solution which contained no Cat0059 compound. This was the control group.

At the end of each incubation period, the eye-formula was discarded and the surface of globe was rinsed with dH2O several times. After rinsing, 400 µL of aqueous humour was harvested from each eye globe using a 500 µL sterilised syringe (Terumo® Syringe, Terumo Co., Philippines). The cornea and iris were then dissected and rinsed with dH2O and weighed prior to storage in a tube. Finally, the lenses were harvested and rinsed with dH2O and the cortical region of the lens (including capsule, epithelium and outer fiber cells) were collected and weighed.
Preparation of samples for HPLC analysis
Aqueous humour was vortexed in 2 mL dH₂O. Lens samples were sonicated in 2mL dH₂O. Corneal samples were cut into smaller pieces and ground into a powder using a hand mortar and pestle under 3-5 mL of liquid nitrogen. The powdered cornea was then transferred into a tube containing 2 mL dH₂O. The homogenisation of the iris followed the same procedure as the cornea. The extraction of homogenates followed the procedure described in Chapter 5 (5.3.2.1.). The final, concentrated extract volume was 100 μL per sample. 10 μL of the extract volume was used for each HPLC analysis.
A standard series of known amounts of Cat0059 (0, 50, 125, 250, 500 and 1500 ng) in 100% ethyl acetate was injected at the same time as the samples (HPLC protocol for Cat0059 refer to 5.3.2.2. in Chapter 5).

6.2.2. Results

Measurement of Cat0059 presence in lens with HPLC
The detection and separation of Cat0059 was satisfactory in the 0 and 1500 ng range. The correlation with the HPLC Peak Area is shown in Figure 6.1(R² = 0.99). The separation of Cat0059 by HPLC was a reliable technique and has the ability to quantitate up to 1500 ng in 5 μL injection volume.

![Peak Area vs. Amount of Cat0059](image)

Figure 6.1 Standard curve of Cat0059 (n = 4)
Detection of unbound Cat0059 from the extracted samples by HPLC

After the first hour of incubating with corneal region with the eye-drop solution containing 3 mM Cat0059, 300 nM Cat0059 was detected in the aqueous humour. Thus, about 0.01% of the Cat0059 penetrated to the aqueous humour through the cornea (Table 6.1.).

After a 2 h incubation period with the eye globe in contact with the Cat0059 solution, there was approximately a 120 fold increase of the Cat0059 in the aqueous humour compared to the 1 h incubation period. However, no Cat0059 was detected in the cortical region of the lens after the 1 h or 2 h incubation periods. After a 24 h incubation period, 100 μM Cat0059 was detected in the aqueous humour and approximately 0.3 μM Cat0059 in the lens. The latter is about 0.3% of the concentration of Cat0059 in the aqueous humour.

The amount of Cat0059 that penetrated into the different parts of the eye globe was calculated after the 2 h incubation period. The total amount of Cat0059 deposited in the three regions of the eye: cornea, aqueous humour and iris was approximately 36 μg.

The distribution was 83% in the cornea, 16.5% in the aqueous humour and 0.5% in the iris. These figures indicate that most of the Cat0059 was trapped within the corneal region and only 17% entered the intraocular environment.

Table 6.1 Measurement of Cat0059 concentration by HPLC after the cornea of the ovine globe was in contact with 3 mM Cat0059 eye-drop formula, for three different periods. (-): results not available. *Concentration was calculated in 500 μL aqueous humour. #Concentration was calculated in 0.5 g cortical region of the lens. Average wet weight used for extraction of cornea = 0.4 g and iris = 0.35 g. Number of globes used in 1 h, 2 h and 3 h incubation were 1, 2 and 2 respectively.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Concentration of Cat0059 detected in samples after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cornea</td>
</tr>
<tr>
<td>1 h</td>
<td>75 μg /g</td>
</tr>
<tr>
<td>2 h</td>
<td>12 μg/mL *36 μM</td>
</tr>
<tr>
<td>24 h</td>
<td>-</td>
</tr>
</tbody>
</table>
6.3. Experiment 2: Cat0059 in a lens culture system

The purpose of this experiment was to examine the cytotoxicity of Cat0059 to the lens and to assess the permeability of the lens to Cat0059. This experiment mimics the eye-drop treatment previously described in the in vivo trials (Chapter 5). The same amount of Cat0059 (40 μg) in a single eye-drop solution was directly added to the culture media for a period of time. The intact lenses were then exposed to a hyper-Ca\textsuperscript{2+} solution containing Cat0059 to evaluate the efficacy of Cat0059 in preventing opacity.

6.3.1 Methods

**Lens culture**
Several pairs of eye globes from the same breed (9 – 11 months old) were removed immediately after the animals were slaughtered. Lenses were dissected within 2 h of death using the posterior approach. All dissected lenses were kept in pairs. A group of lenses (Group A) were cultured in AAH (refer to Chapter 3, 3.2). Another group of lenses (Group B) were cultured in EMEM. The medium was replaced after the first 24 h incubation.

**Preparation of treatment solution**
Cat0059 was synthesised by the University of Canterbury-Chemistry Department and was solubilised in DMSO. The final concentration of DMSO in the culture medium, after addition of the inhibitor, was less than 0.05% v/v in both the control and treated groups. For the experimental period, adequate volume of DMSO was also added to a set of lenses (control groups) incubated in culture medium, AAH and EMEM, so that DMSO does not attribute to inhibitory effect on the lens.

**Experimental Protocol**
After the 1 h pre-incubation period (Group A), 8 lenses (one lens from each pair) were cultured in AAH containing DMSO (as the control group). 8 lenses (the other lens from each pair) were cultured in AAH containing 10 μM Cat0059 (40 μg Cat0059 in 10mL AAH per lens). 4 pairs of lenses were cultured for 100 min using the same protocol. This time was based on the estimated time for a complete change of the aqueous humour in human eyes (Lee
& Robinson, 1986). The remaining lenses were cultured for 5.5 h using the same protocol, to maximise the chances of Cat0059 penetrating the lens.

After the 48 h pre-incubation period (Group B), 3 lenses (one lens from each pair) were cultured in EMEM containing DMSO (as the control group). The remaining 3 lenses were pre-incubated with 10 μM Cat0059 in 0.03 % v/v DMSO for 2 h. After this period 2 M Ca\(^{2+}\) in dH\(_2\)O was added to each of the 6 lenses to make the final concentration 5 mM Ca\(^{2+}\). All lenses were then cultured for 44 h.

_Cytotoxicity of Cat0059; LDH Leakage Assay_
At the end of the 100 min and 5.5 h incubation periods (Group A), 5 ml of AAH medium from each lens was collected and concentrated to 2 mL with a SpeedVac concentrator. These samples were assayed for LDH leakage (3.2, in Chapter 3).

_Stability of Cat0059 in the culture condition_
The stability of Cat0059 in culture media was determined by measuring the amount of Cat0059 remaining in the culture medium after each incubation period (Group A). At the end of the 100 min and the 5.5 h experimental period (Group A), 5 mL of AAH from each lens was collected and concentrated to 2 mL with a SpeedVac concentrator. Cat0059 from the sample was extracted in 100 μL ethyl acetate (the extraction procedure refers to 5.3.2.1 in Chapter 5).

_Uptake of Cat0059 in the lens_
At the end of each experimental period (Group A), all the lenses were harvested and rinsed with water. Epithelium, cortex and nucleus fractions of the lenses were dissected and weighed. Each fraction of the lens was homogenised in 1.5 mL of dH\(_2\)O by sonication. Cat0059 from samples was extracted in 100 μL ethyl acetate (for the extraction procedure please refer to Chapter 5). All the extracted fractions from each incubation group (100 min or 5.5 h) were pooled and further concentrated to 100 μL.

At the end of the 44 h experimental period (Group B), the 6 lenses were harvested and rinsed with water. Epithelium and cortex fractions of the lenses were dissected, homogenised and extracted (Chapter 5, 5.3.2.1). For further concentration of Cat0059, three-parts-volume from each extracted lens sample were pooled and concentrated to 100 μL.
Measurement of the Cat0059 by HPLC
10 μL of extracted samples was injected into an HPLC column for separation and quantification of the amount of Cat0059 in the lens (refer to Cat0059 HPLC protocol, Chapter 5, 5.3.2.2). A series of known amounts of Cat0059 (0 - 250 ng) in ethyl acetate were put through the HPLC as a standard curve at the same time as the samples.

6.3.2 Results

Measurement of Cat0059 in the lens by HPLC
There was no detectable Cat0059 in the lenses incubated for 100 min or 5.5 h.

LDH leakage during experiment
Over the 5.5 h of incubation, LDH leakage levels steadily increased (P < 0.05) in both the control and Cat0059 treated groups. The level of LDH leakage between the control and Cat0059 treated lenses in pairs was not significantly different. This indicates there is no sign of cytotoxicity caused by the Cat0059 treatment during the 5.5 h incubation in the culture system.

Figure 6.2 A plot of mean LDH leakages released (± sd) from ovine lenses (in pairs) during culturing in AAH + 10 μM Cat0059 (Cat0059, capital letter) and AAH (Control, lower case letter) for 5.5 h. Different letters indicate significant differences by Paired t-test (P < 0.05). 8 pairs of lens were analysed at 100 min, while 4 pairs of lens were analysed at 5.5 h.
Stability of Cat0059 in the culture condition

The total amount of Cat0059 remaining in the medium after the period of incubation showed that the different incubation periods did not affect the availability of Cat0059 (Table 6.2).

**Table 6.2** Estimated amount of Cat0059 (± sd) remaining in the culture medium (10 mL) at the end of each 100 min and 5.5 h incubation period. Two-sample t-test (n = 4).

<table>
<thead>
<tr>
<th>Initial Cat0059 (μg) in 10 mL</th>
<th>36.8</th>
<th>36.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lens culture duration</td>
<td>after 100 min</td>
<td>after 5.5 h</td>
</tr>
<tr>
<td>Remaining Cat0059 (μg)</td>
<td>25.1 ± 5.5</td>
<td>26.1 ± 7.0</td>
</tr>
</tbody>
</table>

Lens Transparency and uptake of Cat0059 in 44 h incubation

For the 44 h incubation treatment with Cat0059 in hyper-Ca\(^{2+}\) conditions, opacification was prevented in the lenses incubated with 10 μM of Cat0059.

Despite the visual improvement in lens clarity of the Cat0059 treated lenses, HPLC analysis failed to detect any Cat0059 in either the epithelium or cortical regions of the lens. Analysis of pooled and concentrated samples confirmed that Cat0059 was undetectable by HPLC.

![Figure 6.3](image)

**Figure 6.3** Digital images and mean opacification scores (n = 3, ± sd) of ovine lenses after culture in EMEM (Control), EMEM + 5 mM Ca\(^{2+}\) (Ca\(^{2+}\) only) and EMEM + 5 mM Ca\(^{2+}\) + 10 μM Cat0059 (Ca\(^{2+}\)+Cat0059) for 44 h. Opacification scores: 100 = Full opacity and 1 = clear/transparent lenses. *Paired t-test relative to Ca\(^{2+}\) only lenses (P < 0.003).
6.4. Experiment 3: Bioavailability of Cat0059 in an ocular environment.

The purpose of this experiment was to assess the bioavailability of Cat0059 in two biological intraocular environments, the aqueous humour and the lens homogenate. A standard protein binding assay for the compound was used to assess the interaction between Cat0059 and intraocular compartments (Inoue et al., 2003).

6.4.1 Methods

Aqueous humour and lens homogenate sample preparation

Aqueous humour was collected from 4 normal lamb eye globes within 3 h of sacrifice. 600 μL aqueous humour from each eye was pooled (2400 μL) to eliminate any variation between the individuals. For lens homogenates, six eye globes from the same breed of sheep (9–11 months old) were collected immediately after sacrifice and dissected within 2 h. Epithelium and cortex fractions of the 6 lenses were dissected and weighed separately prior to being placed in separate labelled tubes. Two homogenisation buffers were used in this study: dH₂O or dH₂O with 1mM EGTA. Dissected samples from the first 3 lenses were homogenised with equal volumes of dH₂O (w/v) by sonication. Aliquots of these homogenates were diluted with dH₂O to a final dilution of 1:6. The three diluted homogenates were then pooled together. Dissected samples from the other 3 lenses were homogenised with equal volume of dH₂O containing 1mM EGTA (w/v), by sonication. Aliquots of these homogenates were also diluted with dH₂O containing 1 mM EGTA for a final dilution of 1:6. The three diluted homogenates were then pooled together. The protein concentrations of the pooled aqueous humour and pooled lens homogenates were measured by the BCA protein assay using bovine serum albumin as a standard (refer to 4.2.1, Chapter 4).

Experimental protocol for Cat0059 binding factors

To test for the presence of the aqueous humour binding factors for Cat0059, three aliquots of 300 μL pooled aqueous humour were placed into each of three microcentrifuge tubes and 20 μL of 0.15 mM Cat0059 in 50% acetonitrile/dH₂O solution was added to each tube to make a final concentration of 10 μM Cat0059. Another three aliquots of 300 μL pooled aqueous humours was placed into each of three microcentrifuge tubes and 20 μL of 1.5 mM Cat0059 in 50% CH₃CN/dH₂O solution was added to each tube to make a final concentration of 100 μM Cat0059.
To determine the lens homogenate binding factor, aliquots of pooled lens homogenate samples (300 μL) were fortified with 20 μL of the 0.15 mM or 1.5 mM Cat0059 (10 μM or 100 μM respectively) solution and vortex mixed, as described previously. All microcentrifuge tubes were vortex mixed and incubated in an incubator at approximately 37°C for 20 min.

**Centrifugal ultrafiltration**

After 20 min incubation, the entire contents of the incubates were transferred separately into a Microcon® YM-10, Centrifuge Filtering Device (10,000Da Mₜ cut-off) for centrifugal ultrafiltration. The samples were centrifuged at 14,000 g for 40 min until the volume of retentate (the upper portion of the filtering device, containing compounds larger than Mₜ 10,000 Da) was reduced to approximately 5 μL. The filtrate was then transferred to labelled HPLC vials for the HPLC.

**Non-specific binding of Cat0059 to centrifugal ultrafiltration apparatus**

The recovery of Cat0059 through the centrifugal filtration procedure was measured. Three aliquots of 0.1M phosphate buffer saline (300 μL, 1 x PBS, pH 7.4) were placed into each of three microcentrifuge tubes and 20 μL of 0.15 mM Cat0059 solution was added to each tube to make the final concentration of 10 μM Cat0059. Another three aliquots of 0.1M PBS (300 μL) was placed into each of three microcentrifuge tubes and 20 μL of 1.5 mM Cat0059 solution was added to each tube to make the final concentration of 100 μM Cat0059. All microcentrifuge tubes were vortex mixed and incubated in an incubator at approximately 37°C for 20 min, prior to the centrifugal ultrafiltration procedure. The resulting filtrate was analysed by the HPLC to determine the percent of non-specific binding of Cat0059 to the centrifugal filtration apparatus.

**Evaluation of extraction procedure by the HPLC**

The aqueous humour was collected from an eye globe, immediately after sacrifice. Aliquots of 200 μL aqueous humour were fortified with two different amounts of Cat0059 in 50:50 acetonitrile/dH₂O, vortex mixed and incubated for 1 h at RT. These two samples were subjected to the extraction procedure (5.3.2.1 in Chapter 5) and concentrated to 100 μL. The retentate and pellet fractions of the lens homogenate after the centrifugal ultrafiltration were also collected. These fractions were subjected to the extraction procedure (5.3.2.1 in Chapter 5) and concentrated to 200 μL. 10 μL was analysed by HPLC for the measurement of Cat0059.
**Measurement of Cat0059 by HPLC analysis**

The amount of Cat0059 present in samples was quantitated by the HPLC (Cat0059 HPLC protocol, 5.3.2.2, Chapter 5). A series of known amounts (0 - 500 ng) of Cat0059 in acetonitrile/dH2O was run through the HPLC column as standards.

**6.4.2 Results**

**Protein content**

The average total protein content in sheep aqueous humour was 1.42 ± 0.67 mg/mL (n = 4). Since the variation was large between aqueous samples, 4 aqueous humours were pooled to give a protein concentration of 1.25 mg/mL. Lens homogenates of pooled epithelium and cortex fractions of the lens (n = 6) contained 45 mg/mL total protein content. Therefore, there was 270 mg/mL of total protein content in the cortical region of the lens (the homogenates were diluted by 1/6).

**Binding factor of Cat0059 with aqueous humour**

Non-specific binding of Cat0059 to the centrifugal filtering apparatus was 6.1 ± 0.3% (n = 6). The final binding factor of Cat0059 (Table 6.3) was then estimated by subtracting the non-specific binding factor from the aqueous humour binding factor. The results of the final binding factor were that 0.5% and 14% of 10 μM and 100 μM Cat0059 respectively were bound by substances within the aqueous humour. These results indicated that around 86-99% of the given concentration of Cat0059 remained unbound in the aqueous humour.

**Table 6.3** Portion (%) of Cat0059 (± sd) bound to pooled ovine aqueous humour containing 1.25 mg/mL protein.

<table>
<thead>
<tr>
<th>Concentration of spiked Cat0059 (μM)</th>
<th>Percentage of bound Cat0059 with ovine aqueous humour</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.5 ± 0.01</td>
</tr>
<tr>
<td>100</td>
<td>14.3 ±1.1*</td>
</tr>
</tbody>
</table>

* Two sample t-test indicates significant difference between 10 μM and 100 μM with P <0.005 (n=3).

**Binding factor of Cat0059 with lens homogenates**

After 300 μL of individual lens homogenates were incubated with 10 μM Cat0059, no Cat0059 was detected. Therefore all extracted samples were pooled and further concentrated to increase the sensitivity of the HPLC method. The final binding factor of Cat0059 (Table
6.4) was also estimated by subtracting the non-specific binding factor (6.1 ± 0.3%) from the lens homogenate binding factor.

The results show that 10-100 µM Cat0059 had an estimated binding factor between 75% and 90% with lens homogenates (Table 6.4). These results indicate that around 10-25% of the Cat0059 remained unbound with the lens homogenates containing 45 mg/mL protein concentration. When the binding factor of Cat0059 to the lens homogenate was compared between different homogenate buffers, the binding percentage of Cat0059 was significantly higher in lens homogenates containing 1 mM of the Ca²⁺ chelator, EGTA in dH₂O than in dH₂O only (P < 0.005) (Table 6.4).

**Table 6.4** Portion (%) of Cat0059 (10 µM and 100 µM) bound to pooled ovine lens homogenates, containing 45 mg/mL protein.

<table>
<thead>
<tr>
<th>Homogenising buffer</th>
<th>Bound Cat0059 (%) to lens homogenates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 µM spiked Cat0059</td>
</tr>
<tr>
<td>dH₂O</td>
<td>88.9</td>
</tr>
<tr>
<td>dH₂O+EGTA</td>
<td>91.3</td>
</tr>
</tbody>
</table>

*2-samples t-test (P < 0.005) between two buffers (n = 3).

**Evaluation of extraction procedure**

When aqueous humour was incubated with a known amount of Cat0059, the extraction procedure (Chapter 5, 5.3.2.1) recovered approximately 90% of the added Cat0059, compared to direct injection of a standard Cat0059 onto the HPLC without being subjected to the extraction procedure (Table 6.5).

**Table 6.5** Recovery of Cat0059 after extraction process with ethyl acetate. Pooled ovine lens aqueous humour samples (AqH 1 and AqH 2) were spiked with two different amounts of Cat0059 (0.75 and 1.5 µg) in 50:50 acetonitrile/dH₂O and subjected to the extraction. Two standards (0.75 and 1.5 µg Cat0059 in 50:50 acetonitrile/dH₂O) were also analysed in HPLC with no extraction. Total Peak Area of HPLC chromatography was used to evaluate extraction recovery. Recovery of the extraction procedure (%) = (AqH Peak Area/Standard Peak Area) x 100.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amt of dial in 5 µl</th>
<th>Extraction procedure</th>
<th>* Total Peak Area (UV*sec)</th>
<th>Extraction Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>0.75 µg</td>
<td>no</td>
<td>2,457,624</td>
<td>-</td>
</tr>
<tr>
<td>AqH 1</td>
<td>0.75 µg</td>
<td>yes</td>
<td>2,236,676</td>
<td>91.0</td>
</tr>
<tr>
<td>Standard</td>
<td>1.5 µg</td>
<td>no</td>
<td>5,388,593</td>
<td>-</td>
</tr>
<tr>
<td>AqH 2</td>
<td>1.5 µg</td>
<td>yes</td>
<td>4,807,754</td>
<td>89.2</td>
</tr>
</tbody>
</table>
When the bound fraction (retentate and pellet after centrifugal ultrafiltration procedure) of the lens homogenates were subjected to the extraction process, the HPLC analysis failed to detect the presence of Cat0059. These results suggest that the extraction procedure of Cat0059 with ethyl acetate only extracts unbound Cat0059 in the samples.

6.5. Experiment 4: Intravitreal injection of Cat0059

The purpose of this experiment was to assess the effect of applying the inhibitor directly to the vitreous humour. This was designed to avoid the prime barrier of the eye, the cornea, and to increase the concentration of Cat0059 in the aqueous humour; this should promote the penetration of Cat0059 into the lens.

6.5.1. Methods

Animal sample
One normal lamb (less than 4 months old) was anaesthetised (Lincoln University Animal Ethics Protocol-ACE#109).

Formulation of 0.1% Cat0059 for intravitreal injection
0.1% Cat0059 was solubilized in 10% ethanol and 0.9% NaCl. The solution was filtered with 0.22 μm pore size (PES membrane, Millex® GP, Millipore, Ireland) into a sterilised injection bottle. A single 100 μL injection of the Cat0059 formulation was administrated by Dr Steve Heap directly into the vitreous humour through the sclera just above the iris of the eye (using a sterile 1mL syringe with a sterile needle, Terumo® Syringe, Terumo Co., Philippines).

Sample collection
The lamb was sacrificed 24 h post-injection. Treated and non-treated eye were collected immediately post-mortem. First, aqueous humour (AqH) from each eye was collected by a syringe with a sterile needle (Terumo® Syringe, Terumo Co., Philippines). Then each eye was dissected into lens and vitreous humour (VH) samples. The lens was weighed and homogenised in 3 volumes 4°C Buffer-C (refer to Experiment 3, Chapter 4, 4.5.1) calculated by weight.
**HPLC measurement of Cat0059**

700 μL lens homogenate, 1.3 mL vitreous humour and 220 μL aqueous humour aliquots were extracted in 100% ethyl acetate as described in 5.3.2.1 (Chapter 5). 20 mL of each extracted sample was concentrated to a final volume of 100 μL. 10 μL each sample was injected onto the HPLC column for the measurement of Cat0059 (Cat0059 HPLC protocol, in 5.3.2.2, Chapter 5). A standard curve was constructed from the initial injection solution with 0, 5, 10, 20, 50 and 100 ng Cat0059.

### 6.5.2. Results

The detection and separation of Cat0059 between 0 and 100 ng (5 μL injection volume) was correlated to the HPLC Peak Area as shown in Figure 6.4 ($R^2 = 0.99$).

![Figure 6.4 Standard curve of Cat0059 (0-100 ng).](image)

At 24 h post-injection, approximately 80 ng of Cat0059 in 1.3 mL vitreous humour was detected. The total vitreous humour volume was 3 mL. Thus, the estimated amount of Cat0059 in the entire vitreous humour is 180 ng. As the initial amount of Cat0059 injected was 100 μg, then 0.1% of the injected Cat0059 remained in the vitreous humour 24 h post-injection.
At 24 h post-injection, approximately 170 ng of Cat0059 was detected in 220 μL aqueous humour. The total aqueous humour volume was 410 μL. Thus, the estimated amount of Cat0059 in the entire aqueous humour is 350 ng. This implies a concentration of about 2 μM of Cat0059 is in the aqueous humour.

No Cat0059 was detected in the lens samples at 24 h post-injection.

6.6. Discussion

**Cornea, the biggest barrier for Cat0059 delivery to the intraocular environment**

The cornea is considered to be the major pathway for ocular penetration of topically applied drugs. In this research, the cornea also appears to be the major barrier for delivering Cat0059 to the intraocular environment with topical instillation. Even when there is constant contact between the eye and a solution of Cat0059, the diffusion rate of Cat0059 to the aqueous humour through the cornea was only 0.01% over the first hour and 3.6% by the end of a 24 h incubation period. The latter illustrates that there is an extremely slow penetration rate through the cornea, even with continuous irrigation, which is more effective than a single topical drop installation - the preferred *in vivo* method.

A high concentration of the molecule in the aqueous eye-drop solutions will increase the cornea permeability through the pre-cornea region, the tear film. Cat0059 was designed to be more water soluble than SJA6017, but in reality Cat0059 was less water soluble than SJA6017. A maximum of 1mg/mL Cat0059 was soluble in an aqueous solution even with the assistance of ethanol (14%).

In other research on drug diffusion across the cornea (Zhang et al., 2004), the corneal epithelium was shown to be the main barrier to hydrophilic molecules. For hydrophilic solutes of approximately 150-450 Da, the relative resistance to diffusion through the corneal epithelium, stroma, and endothelium were predicted to be approximately 92%, 1%, and 7% respectively. Lipophilic solutes with the same size range had relative resistance to diffusion of 75%, 18% and 7% respectively. It is therefore essential that any novel compound for topical application must penetrate the corneal epithelium.
The synthesis of corneal permeable calpain inhibitors has been investigated by modifying the structure of SJA6017 (Nakamura et al., 2003). The replacement of the pyridine ring or cyclic hemiacetal of SJA6017 improved transcorneal permeability, but both molecules displayed reduced inhibitory activities against calpains. This study suggests that both water solubility and the active aldehyde group (at P1 site, Figure 5.6.) in the inhibitor structure are two factors that affect the corneal permeability of SJA6017. Cat0059 is based on the structure of SJA6017. It is apparent that future research needs to explore the link between the molecular structure of calpain inhibitors and their ability to cross the corneal barrier whilst retaining calpain inhibitory activities.

Aqueous humour, an ocular compartment with minor bioavailability for Cat0059 to the lens

After passage through the cornea, drugs are distributed in the anterior chamber by aqueous humour circulation, and from the anterior chamber, diffusion into the lens and other ocular tissues (Burstein, 1985). A standard drug protein binding assay to plasma (Inoue et al., 2003) has been successfully adapted in this research as a simple drug bioavailability test. The test was used to determine the pharmacokinetic profile of Cat0059.

Under non-pathological conditions, approximately 10% of Cat0059 was bound to the ovine aqueous humour components (Table 6.3). 90% was unbound and free for diffusion into the lens. The low interaction of the Cat0059 with the aqueous humour was attributed to the low total protein concentrations of the aqueous humour (1.42 ± 0.67 mg/mL). It is unclear why the proportion of 10 µM Cat0059 bound to the aqueous humour was lower than 100µM Cat0059. The opposite would have been expected if an equilibrium existed.

Lens, another major barrier for the bioavailability of Cat0059 to calpain

The pharmacokinetic profile of Cat0059 is influenced by interaction with the high protein concentration and structural components in the lens. For example, the concentration of ovine cortical lens proteins (270 mg/mL) was approximately 180 fold higher than the protein concentration of its aqueous humour and 5 fold higher than the level of serum proteins in other animals. For instance, bovine serum contains approximately 50 - 80 mg/mL of proteins (Johnson et al., 1993). 85 - 90% of Cat0059 bound with diluted lens protein (45 mg/mL) was similar to the standard protein binding profile figures of SJA6017, using mouse, rat, monkey, dog and human plasma (50 - 70 mg/mL protein concentration) (Inoue et al., 2003).
It would be expected that the binding factor of 85-90% for Cat0059 (diluted lens homogenate) would increase when Cat0059 enters the intact lens with 270 mg/mL protein. However, when 1.28 mg/mL aqueous humour and 45 mg/mL lens homogenates were incubated with the same amount of Cat0059, the Cat0059 binding factor was only increased by 9 fold even though there was a 24 fold difference in the protein concentration of the samples. This suggests one possibility that the binding of Cat0059 to the lens might have its maximum/saturated point with a binding factor of 85 - 90%, or another possibility that there might be different binding proteins present in different ocular components. If any of these possibilities were true, then it could be assumed that a portion of Cat0059 that enters the lens from the aqueous humour route would still be available despite its high protein concentration.

The diffusion properties of Cat0059 between the aqueous humour and the lens were investigated by incubating the eye globes in contact with 3 mM Cat0059 solution for 24 h (Table 6.1). The results showed that 0.3% of the initial 3mM Cat0059 solution in the aqueous humour was in an unbound form after a 24 h incubation period. The diffusion rate of unbound Cat0059 between the aqueous and lens environments is, therefore, 0.013%/h. This diffusion rate was calculated assuming; diffusion of free Cat0059 only (assumed that bound Cat0059 was saturated with the lens components), the penetration and distribution of Cat0059 were homogeneous within the cortical region of the lens (50% of lens wet weight) and the aqueous humour stayed unchanged during 24 h.

**Uptake of Cat0059 in the lens culture and its protective effect on lens transparency**

The inclusion of 10μM Cat0059 in the culture medium prevented the development of lens opacification (Figure 6.3). Cat0059 was, however, not detected in the lens. Based on the estimated diffusion rate and the binding factors discussed above, the amount of unbound Cat0059 can be calculated. Assuming that the amount of unbound Cat0059 entering the intracellular lens environment is by diffusion facilitated by passive transport and that no active transport is involved, the diffusion rate is 0.013% per h between the aqueous humour (media) and the cortical region of the lens. With this assumption, at the end of the 44 h incubation period, the cortical region of the lens should contain 55 nM concentration of unbound Cat0059 in a normal lens. 55 nM of Cat0059 equates to approximately 9 ng of Cat0059 available in the cortical region of the lens. If all 9 ng was extracted in the minimum vial containing volume (100 μL) and loaded onto the HPLC column with maximum injection
volume (10 -20 µL), this would yield 0.9-1.8 ng. For an effective detection of Cat0059 on the HPLC, a minimum of 5 ng injectable Cat0059 is needed (Chapter 5, results 5.3.2.2). The 0.9-1.8 ng would be below the HPLC detection limits. On the other hand, 55 nM Cat0059 could meet the concentration of Cat0059 to reach the half maximum enzymatic inhibitory activity evaluated by in vitro assay (IC\textsubscript{50} of Cat0059 = 30 nM). This calculation demonstrates that the effective Cat0059 concentration level to protect the lens from opacification may not be detectable by the current HPLC technique.

Unlike the aqueous humour environment where a drug is assumed to be uniformly distributed, the compound may not be homogeneously available within the cortical region of the lens. For example, the diffusion rate of 0.013% per h to the lens was made on the assumption that the concentration of Cat0059 was uniformly distributed within the cortical region. In fact, a more likely situation is that the highest diffusion rate occurs at the surface of the lens (near to the epithelium) and the diffusion decreases on progression to the inner part of the lens. Kojima et al. (1992) demonstrated the route of drug penetration into the eye and drug distribution pattern in bovine (in vitro culture system) and rabbit lenses (in vivo topical administration). By using a micro lens sectioning and HPLC detection technique, the drug was detected in the surface of the posterior and anterior of the lens and equator. No drug was detected in the deeper cortex or nucleus. The restriction of drug penetration to a particular region of the lens may be explained by recent studies on the fiber cell plasma membrane specific membrane protein, MP20. During fiber cell differentiation, the insertion of the second most abundant lens membrane protein, MP20, into the fiber cell coincides with the creation of a barrier that restricts the diffusion of molecules into the lens core via the extracellular space (Grey et al., 2003; Louis et al., 1989).

In lenses cultured in a hyper-Ca\textsuperscript{2+} media solution, it was observed that the induced opacity was localised on the epithelium and superficial region of cortical fiber cells (Chapter 5 and Chapter 6). Incorporation of very low levels of Cat0059 (0.8 µM) into the culture medium protected the lens from developing opacity under the hyper-Ca\textsuperscript{2+} medium solution (Figure 5.4, Table 5.2 in Chapter 5). If it is assumed that this surface region is 0.1% of 1 g wet weight of a whole lens and that the diffusion rate is 10% (much higher than 0.013%), the concentration of Cat0059 (0.08 µM at equilibrium) in this region would still be non-detectable by the current HPLC technique, but high enough to inhibit calpain activity.
In this chapter, metabolism of the Cat0059 within the ocular environment has not been considered in estimating the diffusion rates. Drug breakdown and metabolism in the eye is probably limited, but representative of phase I (i.e. oxidising and reducing) and phase II (i.e. conjugating) drug-metabolizing enzymes that have been found in ocular tissues (Burstein, 1985; Urtti & Salminen, 1993). In other research, supporting evidence for the occurrence of limited metabolism is that the portion of unchanged drugs being released from the eye into the systemic circulation following local administration is quite significant (Maurice & Mishima, 1984; Mishima, 1981). Cat0059 contains an aldehyde group which forms a hemithioacetal with the active SH group on the cysteine residue of calpain. The chemical reactivity of the aldehyde is considered to be high and the reaction with various substances under physiological conditions may also be high (Nakamura et al., 2003).

**Limitation to the extraction procedure**

The evaluation of the extraction procedure used in current studies showed that ethyl acetate (Robertson et al., 2005) only extracted unbound Cat0059 and was unable to extract the total amount of Cat0059 that had penetrated to the lens. The possibility that a significant amount of Cat0059 actually enters the lens but remains undetected must be considered.

**Cytotoxicity of Cat0059 and stability in physiological aqueous condition**

Cat0059 did not significantly influence the lens viability at a 10 μM concentration over a 5.5 h incubation period (Figure 6.2). Cat0059 was reasonably stable under the current culture conditions over the different incubation periods. A 25% decrease of Cat0059 in the culture medium during the initial 1 h incubation period is probably due to Cat0059 binding to the lens surface.

**Is EGTA an enhancer for bioavailability of the drug?**

There are different binding factors depending on whether EGTA is included or excluded from the buffer when producing lens homogenates. The presence of Ca$^{2+}$ chelator (1mM EGTA) in the lens homogenate reduced the influence of the Cat0059 binding factor (Table 6.4). Consequently, more free Cat0059 was available to the lens.

Cat0059 binds to the active site of the activated calpain for its inhibitory action and the activation of calpain requires Ca$^{2+}$. The difference in the binding factor, in the presence and
absence of EGTA, was 10%. The question is whether the differences are linked in some way to the activated and non-activated forms of calpains. The 10% difference is calculated to be 3 nmol in a total 300 μL lens homogenate. There is approximately 14 mg protein present in the lens homogenate (268 mg/mL x 1/6 DF x 3/10). If we assume 0.1% of total lens proteins are calpains, this only equates to 0.13 nmol (Mw=130,000 g/mol). This indicates that the binding of Cat0059 to activated calpain is not responsible for the 10% increase in the Cat0059 binding factor.

**Ovine aqueous humour protein concentration**

Other studies have reported very low protein contents in the aqueous humour. For instance, the total protein content of the anterior chamber of the aqueous humour was 0.5 mg/mL in sheep (Francois et al., 1958), 0.42 ± 0.04 mg/mL in rabbits (Langford et al., 1997), 0.77 ± 0.09 mg/mL in calves (Pavao et al., 1989) and 0.33 ± 0.06 mg/mL in monkeys (Gaasterland et al., 1979). Other research has shown that carefully drawn surgical aqueous humour samples eliminate any individual variations (0.12 ± 0.02 mg/mL). Samples collected from post-mortem eyes showed a high degree of variability in the total protein content of human aqueous humour (0.56 ± 0.12 mg/mL) (Tripathi et al., 1989). The large protein variation observed in ovine aqueous humour (1.42 ± 0.67 mg/mL) may be due to changes occurring in the post-mortem eyes in the 3 h after sacrifice.

**Intravitreal injections**

The direct injection of Cat0059 into the vitreous humour showed there was some slow release of the drug from the reservoir. The data revealed that at 24 h post-injection, there was a marked reduction in the concentration of Cat0059 concentration in the vitreous humour with only 0.2% remaining. There was, however, 2.2 μM unbound Cat0059 detected in the aqueous humour at 24 h post-injection (Experiment 4).

The turnover rate of ovine aqueous humour is estimated to be one hour based on rat observations (Mermoud et al., 1996). The presence of Cat0059 in the aqueous humour at 24 h post-injection is evidence that the vitreous humour can potentially act as a reservoir of Cat0059. A sustained release of a drug molecule from the vitreous humour could ultimately provide a chance of the molecule penetrating to the lens if the level of drug concentration in the aqueous humour is high and consistent.
Topical administration of drugs in the future

Despite the inefficiency of drug absorption, the topical application of medications is still the most desirable way to treat ocular diseases, including treatment of cataracts with anti-cataract agents, as there are few side effects. The cornea in the intraocular environment can also retain drugs and has the potential to release them to the aqueous humour as a reservoir tissue. This was demonstrated by most of the unbound Cat0059 (83%) being deposited within its tissue in comparison to the aqueous humour and iris (Table 6.1). Some of the commercially available formulations have an extended pharmacodynamic effect. There is an initial release of a large amount of drug, which is taken up and retained in ocular reservoirs like the cornea, and the drug is then slowly released (Burstein, 1985; Havener, 1983; Piercy, 1985). In other studies, coupling a lipophilic component to a more hydrophilic drug has offered a more successful approach where an active moiety can be liberated after passage through the cornea (known as a pro-drug delivery system) (Lee & Robinson, 1986; Maurice & Mishima, 1984).

In the current chapter, the diffusion rates through cornea penetration and lens penetration have been identified as essential factors that influence the potency of anti-cataract agents and their use as therapeutic agents in the future.
Chapter 7: Overall Conclusion and Future Directions

7.1. Overall Conclusion

The absolute clarity of the lens of the eye is vital in the visual system. Unique structural and physiological properties of the lens are tightly integrated with highly ordered protein content to allow the lens to remain transparent. Cataracts are a universal pathological response to a variety of insults to the lens, and ageing is the most common factor for cataractogenesis in humans. In both ageing and cataractous human lenses, the elevation of intracellular \( \text{Ca}^{2+} \) levels (Duncan & Bushell, 1975) and post-translational modification of lens proteins (Hanson et al., 2000) have been reported. While the complete mechanism leading to cataract formation is largely unknown, extensive research using rodent cataract models has shown that the calpain proteolytic cleavage of soluble crystallins, as a result of an elevation of \( \text{Ca}^{2+} \), may contribute products that induce light scattering. Proteolysis has been proposed, therefore, as an underlining mechanism of cataractogenesis in these models (Azuma & Shearer, 1992; David & Shearer, 1993; Shearer et al., 1997).

**Hypothesis (i): \( \text{Ca}^{2+} \) homeostasis is vital for lens transparency.**

This thesis has shown that the elevation of intracellular \( \text{Ca}^{2+} \) concentration is detrimental to maintain transparency in ovine lenses (Chapter 4). Sustained \( \text{Ca}^{2+} \) influx to the lenses caused the development of opacification. The nature of the opacity was characterised by the formation of a turbid fraction and cell death in the lens, reflecting a disturbance in the fine physiological balance of its unique structure. In conclusion, elevated intracellular \( \text{Ca}^{2+} \) leads to cell death in the lens and cataractogenesis.

**Hypothesis (ii): The loss of \( \text{Ca}^{2+} \) homeostasis leads to a malfunction in the regulation of calpain activity.**

The present research adapted methods to determine whether calpain action is involved in cataractogenesis. Methods included: examining whether autolysis of calpains occurred as an indicator that the calpains are active, determining whether polypeptides which are known calpain substrates *in vitro* are cleaved, and using exogenous calpain inhibitors to identify which properties of cells are affected.
There was increased calpain autolysis associated with the progress of opacification, indicating increased calpain activity (Chapter 4). Spectrin and vimentin, which are known to be highly susceptible to calpain cleavage, were degraded in response to a Ca²⁺ flux (Chapter 4). Degradation of spectrin and vimentin was prevented by synthetic calpain inhibitors (Chapter 5). Addition of calpain inhibitors to the hyper-Ca²⁺ medium prevented the opacification and degradation of cytoskeletal proteins. All of these data suggest that there is uncontrolled calpain activation when the lens loses Ca²⁺ homeostasis and Ca²⁺ concentrations increase.

**Hypothesis (iii):** Deregulation of calpain activity is one of the key mechanisms contributing to cataract formation.

The most significant finding of the current research is the importance of the lens cytoskeletal structure to lens transparency. The exposure of ovine lens to a sustained Ca²⁺ influx induced the development of lenses with opacity. The development of opacity was associated with major degradation of the cytoskeletal structure and with limited proteolysis of crystallins. This makes the OLCO model different to other in vivo cataract models where degraded crystallins were the major contribution to lens light scattering. Cytoskeletal elements of cells are well-known substrates of calpains (Shoeman & Traub, 1990), and the importance of the interaction between cytoskeletal proteins and crystallins in maintaining the lens transparency has been suggested by Clark et al. (1999). This thesis supports the idea that cytoskeletal proteins are important in maintaining lens transparency. The present data demonstrate an important role of the cytoskeletal elements, especially in differentiating fiber cells, in maintaining lens transparency.

Proteolysis of cytoskeletal proteins initiated the breakdown of the integrity of the lens structure, which eventually resulted in the destruction of the lens system if the elevation of intracellular Ca²⁺ was sustained. This was confirmed by partial protection of opacification in the OLCO model by adding a calpain inhibitor to the incubation medium. The data suggest that the proteolysis of cytoskeletal elements, by uncontrolled calpain activation, may be responsible for the lens progressing down a pathological pathway. Therefore this studies propose that the deregulation of calpain activity can be an initial trigger for a series of cascading events in the OLCO model. On the other hand, lens opacification induced by the hyper-Ca²⁺ culture medium model involved surface localised light scattering, which was prevented by applying exogenous calpain inhibitors to the incubation medium (SJA6017 and
two novel calpain inhibitors) (Chapter 5). These two Ca\textsuperscript{2+}-overloaded lens models (OLCO model and hyper- Ca\textsuperscript{2+} culture medium model) provide evidence that calpain is possibly one of the key mechanisms in cataractogenesis.

**Potential in the use of calpain inhibitors as anti-cataract agents**

The penetration of calpain-inhibiting molecules into the lens was a major factor limiting the investigation of the involvement of calpains and assessing the efficacy of applying calpain inhibitors to eyes to prevent cataract progression (Chapter 5 and Chapter 6). Topical administration of novel anti-calpain molecules failed to retard the progression of cataract formation in the *in vivo* ovine inherited cataract model (Chapter 5). In Chapter 6, further investigations on drug penetration showed that the permeability of the molecules across the cornea and the ability of the molecules to penetrate to the lens were limited. Consequently their potential as anti-cataract agents is low using the current delivery system. A more sensitive detection method for determining molecules in tissues is also required if the features of their pharmacokinetics are to be understood.

### 7.2. A model for Cataractogenesis:

Taken together, the current thesis suggests a model for cataract formation when Ca\textsuperscript{2+} homeostasis is disturbed in the lens. The mechanism proposed for cataractogenesis is summarised in Figure 7.1, which illustrates the existing research findings (red arrows) and incorporates the new research findings in this thesis (black bold letters).

It is suggested that the process is initiated (1) by the loss of Ca\textsuperscript{2+} homeostasis and the elevation of intracellular Ca\textsuperscript{2+}, which (2) results in deregulation of the calpain system and, consequently, over-activation of calpain. (3) Excessive activation of calpain results in uncontrolled proteolysis of cytoskeletal proteins (calpain inhibitor prevented the degradation of the cytoskeletal proteins and opacification). (4) Proteolysis of cytoskeletal elements of the lens is then accelerated in lens structural homeostasis by further destruction of the lens membrane and a disturbance. (5) Cataracts (the opacity of the lens) are, therefore, a pathological response to the loss of Ca\textsuperscript{2+} homeostasis and the effect of death of the cells in the lens.
Figure 7.1. A proposed diagram of cataractogenesis in response to changes in Ca\(^{2+}\) homeostasis (modified version of Figure 2.1). The confirmation and novel contribution of the present studies to the hypothesis (2.2) are illustrated by bold numbers and letters respectively. Red solid arrows indicate the major findings of the current study that leads to the pathway for cataractogenesis, whilst the red broken arrows indicate other findings that support the pathway of the current proposed model of cataractogenesis.

7.3. Future directions
Calcium ionophores are a valuable tool for manipulating intracellular Ca\(^{2+}\) homeostasis. The characteristics of an increase in intracellular Ca\(^{2+}\) level can differ, however, depending on the type of cells and the ionophore concentration (Galitzine et al., 2005). Unlike single cell line culture studies, the action of Ca\(^{2+}\) ionophores on the lens involves more pathways according to the physiological and biochemical properties of different regions within the lens (physiological zones). There has been limited use of Ca\(^{2+}\) ionophores in the whole lens system. More sophisticated detection methods to measure Ca\(^{2+}\) distribution in response to the
distribution of ionophores are required in future research to obtain a clear understanding of the changes in Ca\textsuperscript{2+} levels in different physiological zones and their linkage to Ca\textsuperscript{2+} ionophore changes.

Studies on Ca\textsuperscript{2+} regulation in specific regions of the lens, particularly in differentiating fiber cells, need to accompany the whole lens studies since the current studies showed that the opacification was limited to differentiating fiber cells during changes in Ca\textsuperscript{2+} homeostasis. One approach is the use of a cell culture system in which the epithelial cells differentiate into lentoids which closely mimic early fiber cells (Churchill & Louis, 2002; Tenbroek et al., 1994). Using this approach, recent studies have revealed a sustained elevation in the resting intracellular Ca\textsuperscript{2+} in the lens fiber cells in normal lens cell differentiation (Churchill & Louis, 2002). Calpain activity is reported to be higher in the cortex region than the nucleus (Robertson et al., 2005; Yoshida et al., 1984). Therefore understanding Ca\textsuperscript{2+} regulation in the differentiating fiber cells associated with calpain would assist understanding the significance of Ca\textsuperscript{2+} homeostasis in these outer shell fiber cells.

Although introduction of calpain inhibitors into cells contributed to determining the cellular function of calpains, the specificity of synthetic exogenous calpain inhibitors has often been a limiting factor in studying the physiology of the calpain system (reviewed by Wang and Yuen, 1998). So far, calpastatin (an endogenously synthesised inhibitor) is the only specific natural molecule to inhibit calpains. To determine the involvement of calpains in cellular events, alternative approaches need to be developed to provide more sensitive biochemical methods for detecting changes in calpain activities in response to insults. For example, the use of selective antibodies, such as the one that specifically labels the calpain-cleaved fragments of the substrate spectrin, have been used in other calpain-associated pathological conditions (Saido et al., 1993b). Development of methods to detect spectrin breakdown before cataracts are visible would help to confirm the role of calpains in cataractogenesis.

In the current thesis, the formation of high molecular weight products by cross-linking was detected. Such aggregation could affect the integrity of the lens structure as much as proteolysis of the cytoskeleton. For a comprehensive understanding of the importance of Ca\textsuperscript{2+} homeostasis in maintaining lens transparency, the role of transglutaminases in association with calpain activation needs to be examined, as they often compete for the same endogenous
substrates. Transglutaminase activity has been observed in the human lens (Lorand et al., 1981). Research has also demonstrated that vimentin, another cytoskeletal element, is also a substrate for lens transglutaminase (Clement et al., 1998). It would be interesting to investigate the interaction between transglutaminase and calpain activities in cataractogenesis.

The opaque region of the cataractous lens is the result of complex pathology of the lens and the loss of lens transparency. Both effects are permanent. This thesis supports the involvement of calpains at an early stage of cataract formation in response to increased cytosolic Ca$^{2+}$ levels. Consequently, prevention of potential cataract formation, even before visual signs, should be a key goal for cataract treatment. The ovine heritable in vivo cataract flock provides a great opportunity to test anti-cataract agents, particularly if the responsible gene is identified and therefore allows prediction of potential cataract lambs.

It is also important to measure the penetration of the novel anti-cataract agents within the eye. Structural modifications to novel calpain inhibitors may include features which will enhance the ability to detect the compounds within the tissues. For instance, inclusion of heavy isotopes as a part of the synthesis of inhibitors (Nagata et al., 2001) or compounds that incorporate a probe that can later be reacted with other agents to produce a feature which would increase the sensitivity of HPLC to detect inhibitors.

For therapeutic success with topical administration, the synthesis of corneal permeable calpain inhibitors must be investigated by modifying the structure of current calpain inhibitors to improve their penetration properties, increase their water solubility and lower their IC$_{50}$. Non-specific binding of calpain inhibitors to other cysteine proteases would not only reduce their efficacy, but could also make it difficult to determine the involvement of calpains in cataractogenesis. Increased specificity of novel calpain inhibitors is needed to explore the role of calpains in normal physiological events. Future tasks are to identify systems that enhance inhibitory efficacy. It is therefore apparent that future research needs to explore the link between the molecular structure of calpain inhibitors and their ability to cross the corneal barrier whilst retaining their calpain-specific inhibitory activity. The application of effective anti-cataract agents to the millions of people worldwide, before the development of cataract, will be the most desirable approach to prevent or slow down cataract formation as a non-surgical therapeutic option.
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Publication & Conference poster presentations arising from this thesis


