Infant Tear Film Collection and Composition

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Summary

The aim of this thesis was to enable collection and examination of the infant tear film. Therefore a new collection method was developed and applied.

Different absorbent materials were tested for the best extraction quality and a suitable material selected for sample storage, sterilisation and for protein analysis. Cellulose rods were the ideal choice and were compared to glass capillary tubes, which are the standard collection method for adult tears.

In adults, tear collection with cellulose rods was efficient and no difference in the mean total and major protein concentrations was found (Bradford test and gel electrophoresis). Safety of the technique was assessed by measuring the serum albumin concentration in the tears. Samples did not show damage to the ocular surface by serum leakage. When applied to premature and full-term infants, the cellulose rod successfully collected samples and was a suitable and sensitive technique for measuring the protein content and concentration for total and major protein assays. It was shown that sample volume sizes increased with post-conceptional age. In contrast to adult tears, no relationship between tear volume and protein analysis was found. However, similar to adult tear proteins a relationship with closed and open eye tears was found. In addition, tear ferning showed mainly grades that indicate a healthy tear film. In a pilot study, the cellulose rod was applied to a small number of paediatric contact lens wearers. Tear collection, transport from the clinical location to the laboratory and analysis of tear samples were successfully carried out. Higher numbers of subjects are necessary for conclusive data analysis.

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1 General introduction and thesis aims

The study of tear secretion in neonates has been neglected due to the assumption that infants' tearing is delayed. Recent studies report a very stable tear film with a thick lipid surface (Kaercher, Mobius and Welt, 1994; Isenberg et al., 2003; Lawrenson, Birhah and Murphy, 2005). To analyse tear secretion sufficiently, tear samples need to be collected from the infant ocular surface and analysed. This thesis will search for new materials to absorb tears and develop a novel tear collection technique which will then be used for analysis of infant tears.

In this first chapter, literature about the tear film, its important function, modelled structure, and various contents, will be reviewed. These factors contribute to the healthy, stable state and properties of the ocular surface. Furthermore, this chapter reviews the background to the development and current knowledge about infant tears and where deficits exist. Finally, it will list techniques commonly used for clinical and biochemical analysis of tears.

1.1 THE TEAR FILM

1.1.1 Functions

The pre-ocular tear film has a number of important roles in supporting the function of the healthy cornea and conjunctiva (Holly and Lemp, 1977; Tseng and Tsubota, 1997; Davidson and Kuonen, 2004). It provides a smooth refracting surface for the ocular optical system, it provides nutrition to the cells of the anterior ocular surface, and it assists in the blink mechanism by providing a lubricant to the movement of the lids.

In return, the lids assist in the formation of the tear film. Lastly, the tear film is involved in the protection of the exposed ocular surface against infection and assists in the removal of unwanted foreign bodies or debris from the anterior ocular surface.

1.1.2 Structure and thickness

The tear film has a complex structure and composition to accomplish these functions. It has a multi-layered structure with three major parts: the lipids, the aqueous tears and the mucous glycoproteins (Holly, 1980). Their sites of origin are the meibomian glands, the lacrimal and the accessory glands, and the conjunctival goblet cells and surface epithelium, respectively.

The different substances in the tear film form in layers and their interaction is essential for the stability of the tear film. The general three-layer model (Mishima, 1965; Holly and Lemp, 1977; Korb et al., 2002) consists of basement mucous layer (0.02-0.04μm), a large aqueous phase (6-9μm), and a lipid surface layer (0.1-0.2μm).

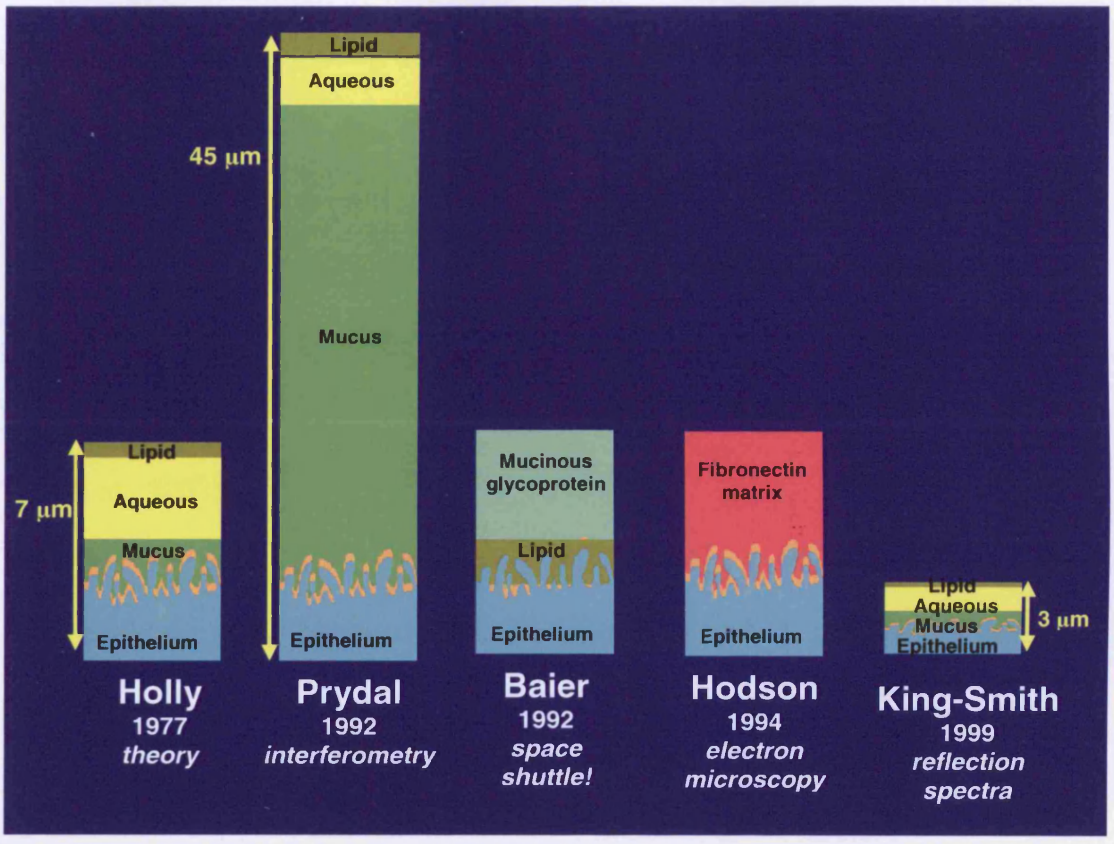


Figure 1.1 Alternative theories of tear film thickness and structure (based on Efron (2004))

Knowing the tear film thickness helps in the understanding of existing tear flow and tear break-up models (King-Smith et al., 2004). A variety of methods have been used to measure the thickness of the entire tear-film. This has been difficult as the tear film thins gradually after blinking and does not spread consistently on the ocular surface (Benedetto, Clinch and Laibson, 1984). Many different measurement techniques have produced a range of values from 3μm (King-Smith et al., 2000) to 40μm (Prydal et al., 1992). Different thicknesses were explained by modifying the structure models (Figure 1.1), such as by replacing the separate mucous and aqueous layer with a mucin gel with decreasing density towards the surface (Dilly, 1994; Chen et al., 1997; Tran et al., 2003).

In addition, the tears contain functional components such as proteins, enzymes, and ions. According to their function and source of secretion, these components are located in the different layers (Sack et al., 2000).

1.1.3 Layers and Components

1.1.3.1 Mucous layer

Ocular surface defence begins locally in the tear film and the main component of the mucous layer, mucin, plays an important protective role (Watanabe, 2002; Gipson and Argueso, 2003). Mucins are highly glycosylated proteins, i.e. proteins with added sugar chains, and are at least 50% carbohydrate by mass (Gipson and Argueso, 2003). Their molecular weight exceeds 200 kilo Daltons (kDa). They are found in animal or human body fluids and as membrane proteins. Other glycoproteins include enzymes, protein hormones, antibodies, complement factors, blood components, and membrane

proteins (Scott and Eagleson, 1988). Until recently it was believed that the mucous was only secreted by the goblet cells of the conjunctiva, but some mucin genes are expressed by the ocular surface epithelia (Gipson and Inatomi, 1998; Dartt, 2002). Some glycoproteins are found to originate from the human lacrimal gland (Chao et al., 1980; Jumblatt et al., 2003) and the accessory glands (Seifert and Spitznas, 1994). They exist only in a low content and may only have the purpose of serving the epithelia surface of the glands (Gipson and Inatomi, 1998; Gipson, 2004).

The mucous component protects the ocular surface against foreign bodies, invasive bacteria and viruses. In addition, mucous secreted from the superficial epithelial cells of the conjunctiva contributes to the glycocalyx, which ensures the wettability of the epithelia surface (Steuhl and Knorr, 1990). Mucous in the glycocalyx contains a category of mucins called trans-membrane mucins that can be soluble or in a membrane associated form (Carraway et al., 2002). They form a tight barrier and regulate hydration. The second category of epithelially produced mucins is the secreted mucins that have a gel-forming function in the tear film. All these mucins have been assigned a number depending on the order of their molecular characterisation, starting with MUC1. According to the human genome nomenclature (http://www.gene.ucl.ac.uk/cgi-bin/nomenclature/searchgenes.pl), to this date 17 human mucin genes have been found. These are MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC12, MUC12, MUC13, MUC15, MUC16, MUC17, MUC19, and MUC20.

Mucous secretion is generally investigated by impression cytology (Pflugfelder et al., 1997; Murube and Rivas, 2003; Yeo et al., 2003; Versura et al., 2006). Conjunctival

goblet cells for analysis are obtained by the contact of a filter paper with the ocular surface. One of the possible reasons for dry eye might be related to mucous deficiency (Danjo et al., 1998; Argueso et al., 2002). A decreased goblet cell density has been shown to correlate with a reduced break-up time (Marquardt and Wenz, 1980). Pflugfelder et al. (1997) investigated patients with disorders associated with aqueous tear deficiency. It has been shown that mucin deficiency is associated with decreased bulbar conjunctival goblet cell density, but that the same correlation did not exist for patients with meibomian gland disease. The assumption was that aqueous tear deficiency can increase the mechanical stress caused by blinking and alter goblet cell numbers.

1.1.3.2 Aqueous Layer

The mean tear volume on the ocular surface is 6.2±2µl (Mishima 1966). The aqueous layer forms the majority of this tear film volume. Its presence determines the interactions between the lipid layer and the innermost mucous layer, and has an important function in washing away foreign bodies and defending the ocular surface against infections by containing various proteins (Gachon et al., 1979). The aqueous also provides the nutrition in the form of glucose (Schutte, 1977; van Haeringen and Glasius, 1977) and provides the environment for growth factors repairing the ocular surface cells (Klenkler and Sheardown, 2004).

The aqueous is secreted mainly by the lacrimal gland, and the accessory glands of Krause and Wolfring (Milder, 1987). The lacrimal gland contains eosinophils, lymphocytes, macrophages and plasma cells (Wieczorek et al., 1988), which contribute by expression of immunoglobulins and other antibodies, to the immune

function of aqueous. Water, proteins and electrolytes are secreted by the lacrimal gland under the regulation of androgens (Dartt, Hodges and Zoukhri, 1998). Androgens are known to suppress lacrimal inflammation and their deficiency in ageing, menopause and Sjögren's syndrome is known to lead to dry eye (Sullivan and Edwards, 1997). Lacrimal gland secretion is the function of both the acinar and ductal cells in the lacrimal gland.

1.1.3.3 Proteins

Proteins can be divided into different groups, depending on the production site, e.g. serum or locally produced proteins. Another group are antibodies in the tears, immunoglobulins that can be very specifically built to serve the ocular surface. Finally, proteins with a very low molecular weight, such as cytokines will be described. They regulate many different important functions.

- Locally produced and serum originated proteins

In the early 20th century, Lindahl demonstrated antibacterial enzyme action in tears, which was later referred to as lysozyme (Allansmith, 1982). Numerous other tear proteins and their functions have been studied since then. Some proteins have defensive roles such as lysozymes, while others, such as transferrin and albumin, transport minerals and lipids; others, like lactoferrin and tear lipocalin, have an important role in the lipid-aqueous interface (Miano et al., 2002).

Total non-stimulated tear protein has been determined to be between 7.1 and 17.2 μg/μl (Coyle and Sibony, 1986; Fullard, 1988; Sack, Tan and Tan, 1992; Ng, Cho and

To, 2000), depending on aqueous production and secretion. In comparison, blood has a total plasma protein of about 70-75 μg/μl (Harper, 1985). It is no surprise that antibodies appear to be derived principally from serum of the conjunctival blood vessels. However, other tissues, such as the ocular glands and cornea, have also been shown to possess the capacity for protein production (Allansmith, 1982). Therefore, the major tear proteins in the aqueous are differentiated by their source of secretion: for example lactoferrin, lysozyme and tear lipocalin are lacrimal gland proteins, whereas albumin and transferrin are serum proteins. In contrast, another protein produced by the lacrimal gland, Immunoglobulin A, has been found in the mucous layer (Mestecky et al., 1978).

- Immunoglobulins

Immunoglobulins (Ig) are antibody molecules, mostly produced in the lacrimal gland. The blood vessels of the conjunctiva can provide a second source so that, during inflammation, the immunoglobulin levels reach serum levels. An understanding of their chains and their weight is important for identifying and characterising each immunoglobulin. Five sub-classes of immunoglobulins, namely IgA, IgD, IgE, IgG, and IgM exist, of which IgA is the most prominent. The tear film contains two different forms of IgA. These are serum and secretory. Secretory IgA is also found in other mucous secretions. Both sub-classes differentiate in their form and molecular weights (Kemeny, 1991). Secretory IgA is considered the first line of mucousal defence (McClellan, 1997) and its concentration is found to rise in a diseased eye (Sen and Sarin, 1979). IgG with its four different sub-classes, is the major component of immunoglobulins in serum. In a newborn, all four sub-classes are transported from the mother and provide protection to the entire body for the first 3 months of life, until

the child develops its own source (Kemeny, 1991). In the tears, IgG has its source from serum and its concentration decreases with non-invasive stimulation (Fullard and Snyder, 1990). IgE has the lowest concentration, in serum, of all the immunoglobulins (Allansmith, 1982). However, it plays a central role in inflammatory and immediate allergic responses in the mucous environment. Measurement of IgE in tear fluid provides a better understanding of allergic diseases of the conjunctiva, such as vernal keratoconjunctivitis (Ebihara et al., 2002). The remaining two immunoglubolins, IgD and IgM, are far less abundant in tears. In blood, IgD appears on the surface of some lymphocytes (Allansmith, 1982). A study by Sen and Sarin (Sen and Sarin, 1979) examined the immunoglobulin levels of the diseased ocular surface and detected no IgD in the tears. In contrast, IgM is only present in the event of infection, and then only for a short time. According to Allansmith (Allansmith, 1982), IgM is the only immunoglobulin not present in the cornea and is therefore less likely to be found in the tear film. However, sensitive protein analysis methods are able to detect small concentrations in tears (Fullard and Snyder, 1990).

- Cytokines

Cytokines are proteins with a low molecular weight of less than 30kDa (Goldsby et al., 2003) that are produced in response to a stimulus and short lived. They regulate various functions such as cell growth and activation, immunity and inflammation, tissue repair and fibrosis. The same kind of cytokine can have a different role in different tissues and organs. White blood cells or leukocytes are the main producer of cytokines (Nicola, 1994).

Their most important function for the ocular surface is the control of inflammation. Observed mucin threads in the inflamed ocular surface led to the assumption that up-regulated cytokines enhance trans-membrane mucin production (Hibino and Watanabe, 2002). However, cytokines on the ocular surface can be harmful. In Sjögren´s syndrome, increased amounts of cytokines may be due to the stimulation caused by the dry environment and result in epithelial damage (Jones et al., 1998).

1.1.3.4 Electrolytes

The tear film contains sodium, potassium, magnesium, calcium, chloride, bicarbonate, and phosphate ions. In contrast to human blood levels, higher concentrations of potassium and chloride are found in the tear film. This suggests a higher concentration and secretion by the lacrimal gland (Milder, 1987). Electrolytes support the ocular surface indirectly by maintaining tear osmolarity within normal limits. Tear fluid ion levels are dependent on the absorbance of electrolytes by the ocular surface by ion channels in the corneal epithelia. In response water is secreted onto the ocular surface (Dartt, 2002; Levin and Verkman, 2004).

1.1.3.5 Lipid layer

Lipids are produced by the meibomian glands within the tarsal plate of the lids. The lipid layer is composed of hydrocarbons, diesters, wax esters, sterol esters, triacyl glycerides, free sterols, fatty acids and polar lipids (Nicolaides et al., 1981). Since the principal lipids, wax and sterol esters, are hydrophobic, it was suggested that free acids are needed to help them spread. This complex composition is important in maintaining a low lipid melting point (Tiffany, 1987) and for spreading of the

hydrophobic lipids (Nicolaides et al., 1981). Interaction with proteins and lipids from the lacrimal gland maintains tear surface tension (Tiffany, Winter and Bliss, 1989). The role of lipids is to form a superficial barrier and slow down evaporation of the tear film (Craig and Tomlinson, 1997). The lipid layer is also a barrier to the skin oils, which can disrupt the tear film (McDonald, 1968). Finally the lipids have a smoothing effect on the blink action (Bron et al., 2004).

The lipid layer is a multi-layer with different phasic polarities (McCulley and Shine, 1997). Temperature, composition, and compression caused through blinking contribute to the formation of the lipid multi-layers (McCulley and Shine, 2001; Bron et al., 2004). The viscosity differs from the aqueous-mucous phase, so that the inner layers flow more rapidly under the lipid layer.

Subjects with meibomian gland dysfunction have a significantly elevated evaporation rate compared to healthy control subjects (Goto et al., 2003). Clinical examination of the lipid layer thickness can be assessed by considering its interference colour (McDonald, 1968; McDonald, 1969). A thicker lipid film is associated with increased tear stability (Nichols et al., 2002). Meibomian gland function can be assessed by observing the lipid of the lid margin, the orifices of the glands and by squeezing the lid margin to test expressibility (Norn, 1987a; Chew et al., 1993; Mathers, Lane and Zimmerman, 1996). The lipid thickness and viscosity can be observed under magnification (McDonald, 1969) (Figure 1.2).

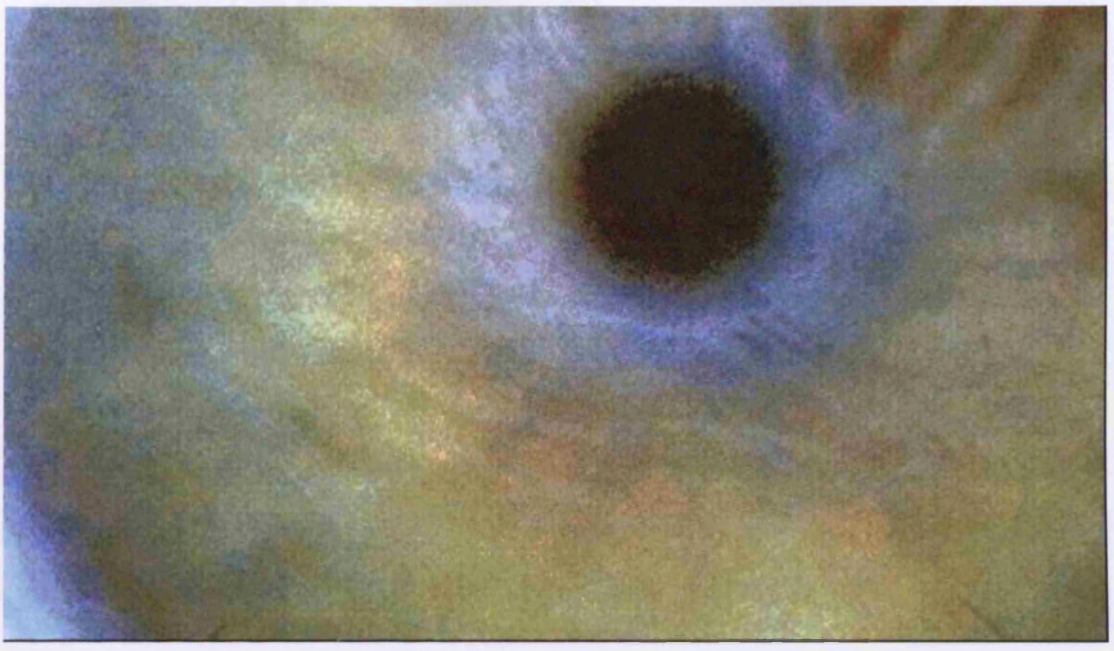


Figure 1.2 The most commonly observed lipid appearance is a wavy reflection (illustrated by Efron (2002a))

For further testing, lipids are collected with a simple technique. Oil from meibomian glands on the lower lid margins is blotted by a tape attached to a meibometer. The lipid on the tape surface is scanned for lipid density by a photo-sensor (Chew et al., 1993a; Yokoi et al., 1999). In addition, to separate the different lipids, chromatography can be used (McCulley and Shine, 1997).

The tear fluid with its previously described structure and contents displays a strong and resistant film on the ocular surface. The next section will have a closer look at the stability and surface tension as forces to maintain it.

1.1.4 Stability and tear break-up

Holly (1985) proposed that the tear film has a thickness of 10 μm and suggested that if the tear film thickness decreases to 1μm, local lipid-mucous contamination will occur, followed by tear break-up.

Many kinds of problem with the tear film can result in instability (Pflugfelder et al., 1998). An unstable tear film shows gaps and breaks, with thinning shortly after a blink. Measurement of the tear break-up time (described later in this chapter) is the only method for stability evaluation. Tear break-up time is expected to decrease with age (Patel and Farrell, 1989) and contact lens wear (Guillon et al., 1997), which alter the lipid and aqueous layers (Hamano et al., 1990; Mathers et al., 1996) and so the tension properties of the tear film surface.

1.1.4.1 Surface tension

The stability of the tear film is dependent on surface tension and is greatest when the surface tension is low; at high surface tensions the tear film tends to form droplets, which prevents spreading on the ocular surface and leads to tear break-up.

Surface tension in a liquid is produced by the attraction between molecules seeking to reduce the exposed surface to the smallest possible area. Water at 20°C has a very high and stable surface tension (Scott and Eagleson, 1988). In contrast, the surface tension of tear fluid is variable and is dependent on the surface area. It is lowest when compressed and comparable to water when expanded (Zhao and Wollmer, 1998). Nagyova and Tiffany (1999) also found a low tension for stimulated tears. Certain tear components, they suggested, are responsible for a lowering effect, in particular

lipocalin and polar lipids. The interaction of meibomian lipids with mucin (Holly, 1973) lowers the surface tension even further (Zhao and Wollmer, 1998). When compared to tear proteins, Tiffany (1989) showed that small amounts of mucin had a greater effect on lowering the surface tension.

1.1.5 Production and secretion of human tears

1.1.5.1 Tear flow

Tears on the ocular surface are referred to as basal or reflex, depending on their production and secretion rate. Basal tears are defined as 'that part of the tears which are secreted when no stimulation is present' (Jordan and Baum, 1980). Under normal basal tear flow conditions, the tear turnover rate averages 1.2 µl/minute with a range of 0.5 to 2.2 µl/minute (Mishima et al., 1966), but when stimulation is reduced by anaesthetics, tear flow decreases to 0.3 µl/min (Jordan and Baum, 1980). Other external factors can influence tear flow, for example, both psychological stimulation (Mathers, Binarao and Petroll, 1993) and direct physical stimulation of the ocular surface can trigger reflex tearing.

Most of the basal secretion comes from the ocular surface (Dartt, 2002). The source of reflex tear secretion is the lacrimal gland. Reflex tears are stimulated aqueous tears, which have abundant amounts of cytokines (epithelial growth factors) and Vitamin A; both of these have an important effect on the ocular surface in dry eye (Tsubota et al., 1999). If reflex tearing is reduced after partial lacrimal gland removal, dryness of the ocular surface such as keratoconjunctivitis sicca (KCS) can occur. This

implies that basic secretion by the remaining lacrimal gland and the accessory glands is insufficient to maintain corneal integrity (Scherz and Dohlman, 1975).

The glands are stimulated to reflex tearing by mechanical, thermal, chemical or light stimuli that follow the depicted pathway in Figure 1.3 (Gupta, Heigle and Pflugfelder, 1997; Stern et al., 1998; Dartt, 20012004). A similar route is assumed for the stimulation of mucous and lipid secretion (Diebold et al., 2001; LeDoux et al., 2001).

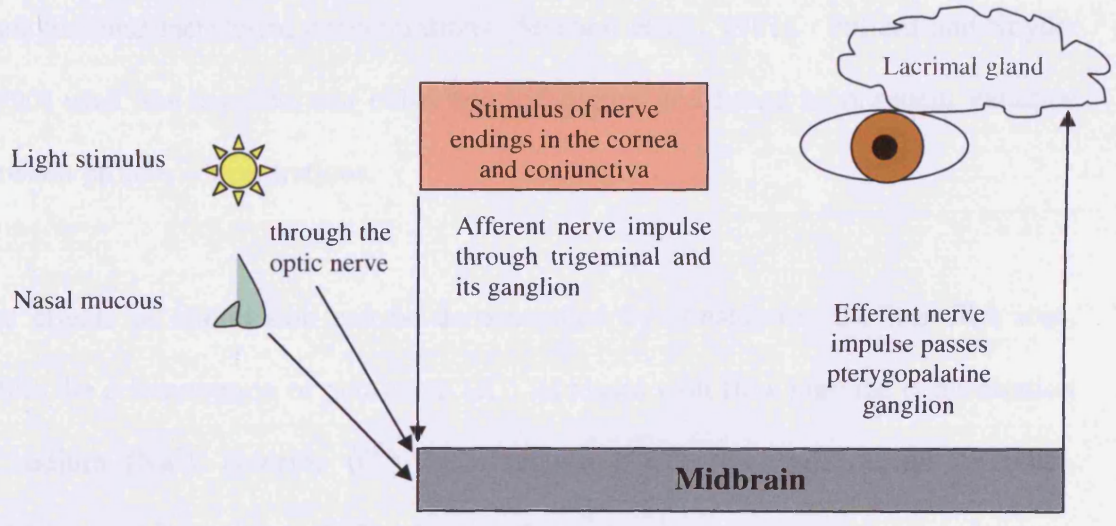


Figure 1.3 The stimulus loop for aqueous secretion

If certain analysis techniques demand large tear sample amounts, tear secretion can be provoked by non-contact means, such as onion vapour or bright light. Once reflex tears are produced by mechanical irritation, significant amounts of serum albumin, gamma globulin, and transferrin become measurable (Josephson and Lockwood, 1964). Usually reflex tears are undesirable, since they show large qualitative and

quantitative variation in their composition (Farris, Stuchell and Mandel, 1981; Stuchell, Farris and Mandel, 1981; Nelson and Wright, 1986; Fullard and Snyder, 1990). An additional disadvantage of reflex tearing is the dilution effect that can mask concentration changes in constituents that are found in small amounts (van Setten et al., 1990). The variation in individual resistance of cornea and conjunctiva cells to trauma could be the reason for this high variability of reflex tearing among individuals.

Basal and reflex secretions have been found to have significant differences in both lysozyme and lactoferrin concentrations (Stuchell et al., 1981). Fullard and Snyder (1990) used non-invasive tear collection techniques and found inconsistent variation between protein concentrations.

The effects of stimulation can be demonstrated by considering the tear film ions. While the concentration of potassium (K^+) increases with flow rate, the concentration of sodium (Na^+), chloride (C^{l-}), and calcium (Ca^{2+}) does not change (Botelho, Goldstein and Rosenlung, 1973). At slow flow rates, ion concentrations may decrease due to higher epithelial absorbency.

1.1.5.2 Blink mechanism

The blink has an effect on the physical nature of the tear film and it directly influences secretion and drainage of the tear film (Tsubota and Nakamori, 1995; Sahlin and Chen, 1997). Spontaneous blinking affects tear spreading and is an active drive of tear drainage through the nasolacrimal duct system (Figure 1.4). Although mechanisms of control are not completely understood, the role of the central nervous

system has been established (Karson, 1989; O'Connor and Petruzzello, 1992; Schmidtke and Buttner-Ennever, 1992) by experiments using visual or cognitive tasks (Tsubota and Nakamori, 19931995; Tsubota, Toda and Nakamori, 1996). Ocular surface reflexes, and environmental factors that affect them, are thought to be the principal determinants of the blink rate (Tsubota, 1998).

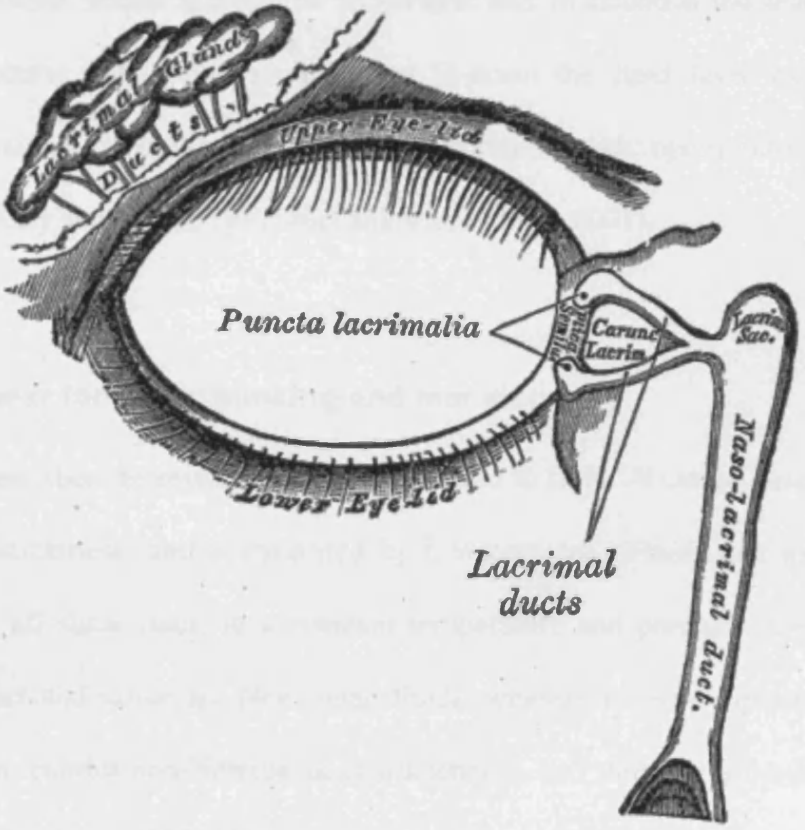


Figure 1.4 Tear drainage through nasolacrimal duct system (illustrated in Gray's Anatomy (Gray and Lewis, 2000))

The 'blink rate' is analysed by counting the number of blinks over a certain time within a certain activity. Normal blink rates in adults vary between 12 and 20 blinks per min, or every 3-5 seconds (Tsubota, 1998; Zaman, Doughty and Button, 1998). An abnormally increased blink rate is measured in dry eye where the ocular surface stimulates more frequent blinking, which is thought to compensate for diminished tear volume (Nakamori et al., 1997).

A frequent blink action is essential to prevent and re-establish the tear film when thinning occurs. The blink is suggested to form the lipid layer by mechanical compression (lid closure) and subsequent expansion (lids open) into the tri-layer structure (Holly and Lemp, 1977; McCulley and Shine, 2001).

1.1.5.3 Shear forces by blinking and tear viscosity

Viscosity describes the resistance of a gas or fluid to flow. It can be described as the quality of 'stickiness' and is measured by a viscometer. Fluids that have constant viscosity at all shear rates, at a constant temperature and pressure, are Newtonian fluids. Water and saline are Newtonian fluids, whereas mucous substances such as the tear film, exhibit non-Newtonian characteristics and thinning according to shear forces (Pandit et al., 1999). The viscosity of Newtonian fluids is not dependent on shear rates or time, although temperature is an influencing factor. At a temperature of 25°C, water has a viscosity of 0.895 mPa.sec (McDonald, 1968).

Even though the main component of tear fluid is water (Gorla and Gorla, 2000), it is a non-Newtonian fluid (Tiffany, 19911994; Pandit et al., 1999) that adjusts to changes in flow by exhibiting 'shear-thinning'. Shear-thinning reduces the tear film's

resistance to movements. The highest shear forces occur during blinking; this allows a rapid reforming of a stable tear film. The maximum shear stress that is produced during blinking is from moving the lipid layer, while the layers close to the ocular surface are stable.

The relationship between shear forces and tear flow can be demonstrated by the viscosity. Tiffany (1991) demonstrated a viscosity of 5 mPa.sec at low shear rates and 1.5 mPa.sec at high shear rate for normal tears, and found no difference between stimulated and non-stimulated tear (Tiffany, Pandit and Bron, 1998). It is uncertain which component in tears contributes the most to this property. Tear proteins, when tested separately, have low viscosity values of 1.3 mPa.sec or less, and exhibit no shear-thinning (Pandit et al., 1999). A comparison with tear mucin and hyaluronan viscosities suggests that tear viscosity may depend on the combination of several macromolecules (Millar et al., 2006).

1.2 EXAMINATION OF THE TEAR FILM

Many examination techniques for the tear film have been developed to help clinicians to determine ocular surface health (Bron, 2001; Yokoi and Komuro, 2004). In the healthy eye, all of the different tear components are maintained within normal parameters. Any disturbance, excess, or deficiency in these parameters can result in 'dry eye'. The exact diagnosis is based on finding specific clinical signs and symptoms. Although dry eye is caused by many factors altering either the quality or amount of tear components, it generally results in disturbed tear stability and epithelial cell damage. Research aims to investigate, with a set of laboratory based methods, the reasons for the disturbances and the effects of any treatment. This is in contrast to the clinical methods.

Many different tear properties and functions are examined and used for differentiating healthy and dry eye conditions. Some are described here.

1.2.1 Integrity of the mucin layer

Reduced protective activity from proteins is associated with a reduced number of goblet cells and a disturbed mucin layer, which determines the severity of the disease (Murube and Rivas, 2003). Disruption of the mucin layer may decrease wound healing in an inflamed dry eye. This may be because accelerated wound healing of the corneal epithelium was observed after application of a 0.3% mucin ophthalmic solution (Shigemitsu, Shimizu and Ishiguro, 2002).

1.2.2 Tear secretion

Hormonal and nervous mechanisms regulate secretion of the different tear components. An impaired hormonal control or neuronal innervation leads to reduction of tear film secretion (Zoukhri, 2006). Even lower reflex tearing secretion is hypothesised to affect the supply of important components such as growth factors and Vitamin A (Tsubota et al., 1999). A higher incidence for dry eye in menopausal women indicates the importance of hormonal regulation.

1.2.3 The solute concentration in tear fluid (osmolarity)

In a dry eye disease, such as keratoconjunctivitis sicca (KCS), the osmolarity is increased (Gilbard, Farris and Santamaria, 1978) and a high variability with each measurement is demonstrated (Farris, Stuchell and Mandel, 1986). Measuring the osmolarity is a highly sensitive and specific diagnostic tool (Farris, 1994) for KCS, but it requires tear collection and expensive equipment. Elevated evaporation, reduced tear secretion, and/or increased tear drainage affect the solute concentration. In a clinical setting, the examination of tear secretion and flow is less invasive and can be used as an alternative.

1.2.4 Tear film stability

Mucous macromolecules in the aqueous that interact with the lipid layer, affect the physical properties, such as the surface tension of the tear film (Nagyova and Tiffany, 1999). In dry eyes with a reduced aqueous secretion, those properties cannot be maintained and tear film stability is lost.

1.2.5 Protein concentration

Finding an alteration in tear protein concentration is useful in the evaluation of ocular surface health. For example, lactoferrin levels have been shown to be reduced in KCS and Sjögren's syndrome (Boukes et al., 1987; Ohashi et al., 2003). Conversely, tear serum albumin levels increase in the reflex tear film of dry eyes, but not in healthy eyes (Farris et al., 1986).

1.2.6 Lipid secretion

The lipid layer minimises evaporation from the tear film surface (Mathers, 2004). Meibomian gland disease alters lipid volume and composition, which results in evaporative dry eye (Gilbard, 1999; Mathers and Choi, 2004).

Some aspects of the tear film and alterations are correlated with each other and usually it is recommended to use more than one test when a diagnosis is needed (Wang, Fukuda and Shimomura, 2005).

1.2.7 Blink rate

The neuronal stimulation of blinking might be linked to the sensation of the ocular surface and stability of the tear film but, according to Tomlinson, Craig and Lowther. (1998), homeostatic regulation is more likely and there is no direct linkage between physical tear parameters.

1.2.8 Methods of observation

1.2.8.1 History

Taking the subject's history has a high value for both the clinician and researcher (Gilbard, 1985; Korb, 2000; Turner, Layton and Bron, 2005). An initial documentation of the patient's history will include common complaints, use of drugs, contact lens wearing and the presence of a disease. For research purposes, the history can be used to exclude subjects with dry eye symptoms or systemic diseases that compromise the tear film from the healthy control group.

In addition to a brief history, questionnaires are used for checking key symptoms and systemic diseases (Glasson, Hseuh and Willcox, 1999; Yeo et al., 2003; Sorbara et al., 2004). McMonnies developed a standard questionnaire for screening, analysing and categorising symptoms mentioned in the history (McMonnies, 1986; McMonnies and Ho, 1987; McMonnies, Ho and Wakefield, 1998).

1.2.8.2 Biomicroscopy

In a routine eye examination the ocular surface and tear film are inspected with a biomicroscope (Korb et al., 2002; Matsumoto et al., 2004). The biomicroscope can also be used to illuminate the area from which tear film is collected, although this approach results in increased tear flow (White, Benjamin and Hill, 1993).

A slit-lamp examination of the tear film and the anterior eye starts with the outer appearance, such as lids, lashes, glands, and blink movements. Only then are the tears and the presence of particles in tears determined. Finally the ocular surface is

examined. Additionally the ocular surface may be stained to view damaged epithelial cells with dyes such as fluorescein (Lemp and Hamill, 1973), lissamine green (Kim and Foulks, 1999) and rose Bengal (Feenstra and Tseng, 1992). This is usually done after contact lens fitting (Orsborn et al., 1989; Edrington et al., 1999; Mountford, Cho and Chui, 2005) or when dryness effects on conjunctival and corneal epithelial cells need to be graded (Bron, Evans and Smith, 2003; Dogru et al., 2005; Versura et al., 2006) (Figure 1.5). Several tests exist in combination with the biomicroscope to observe the stability of the tear film. They are based on characterising the appearance and grading it accordingly.

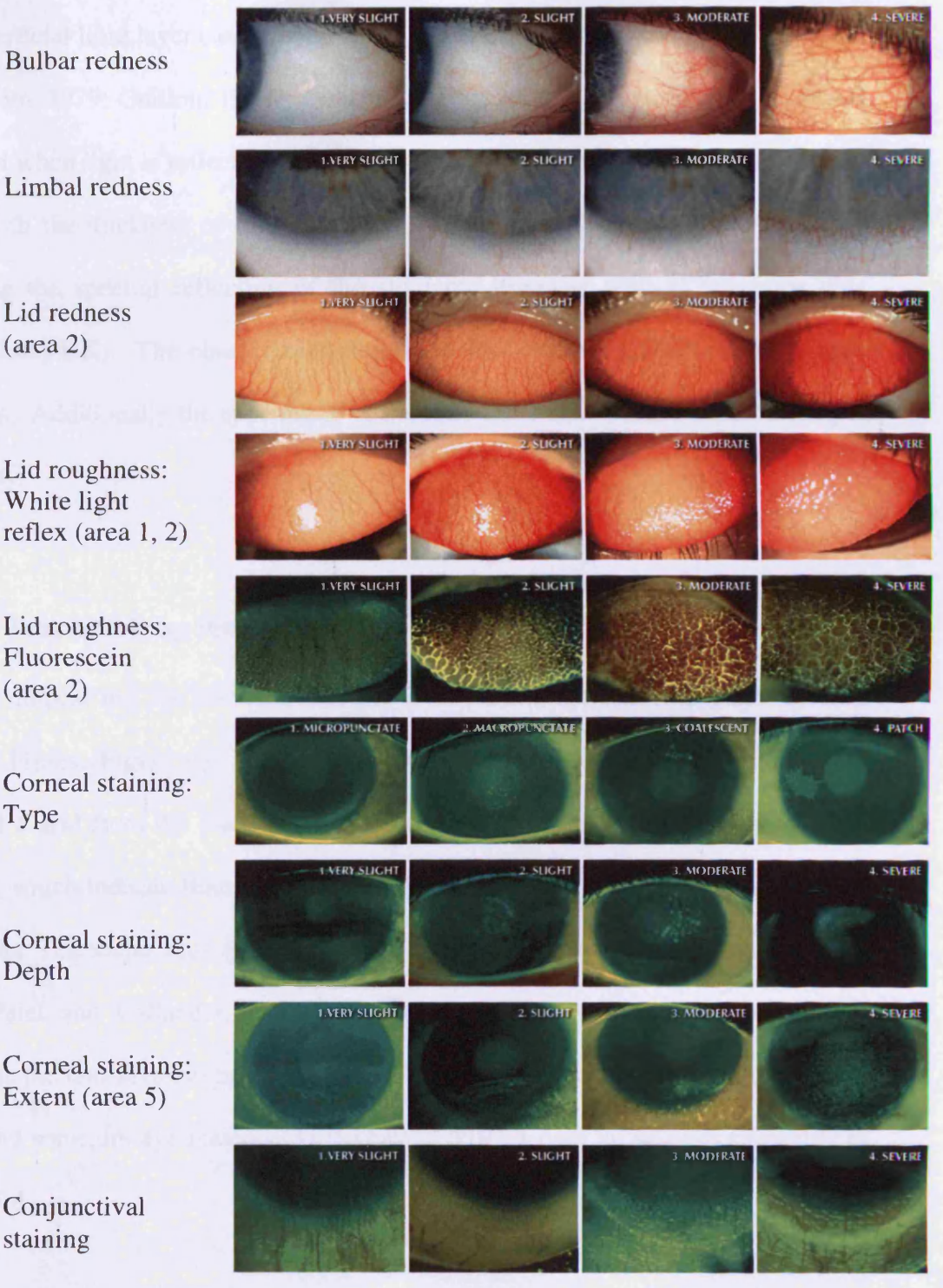


Figure 1.5 The CCLRU grading scale poster was developed by the Cornea and Contact Lens Research Unit (CCLRU) of the University of New South Wales, Australia and is used for assessment of the ocular surface health in contact lens wearers. This copy is sponsored by Vistakon, a division of Johnson and Johnson.

1.2.8.3 Lipid layer interferometry

The superficial lipid layer can be assessed by the interference phenomena (McDonald, 1969; Norn, 1979; Guillon, 1998a; Goto and Tseng, 2003). An interference pattern is produced when light is reflected from the surface of the lipid and aqueous layer and it varies with the thickness of the lipid layer. Interferometry can be done simply by observing the spectral reflection of the slit lamp beam or with a Tearscope Plus (Keeler Ltd., UK). The observed interference colour corresponds to a specific lipid thickness. Additionally the appearance of the lipid layer can be assessed according to its density.

1.2.8.4 Tear break-up time

An easy clinical test for tear film stability is to look at the tear film break-up time between blinks (Figure 6). After a complete blink a reflection, e.g. the reflected image of a grid from the tear film, is observed until distorted or discontinuous lines are seen, which indicate thinning or breaks (Mengher et al., 1985b). Devices, such as the Keeler Tearscope Plus (Guillon, 1998b) (Figure 1.6) or modified keratometers (Hirji, Patel and Callander, 1989) are designed to improve the grid image and determine the non-invasive break-up time (NIBUT). The majority of healthy subjects (62%) and some dry eye patients (37 %) have a NIBUT over 20 seconds (Mengher et al., 1985b).

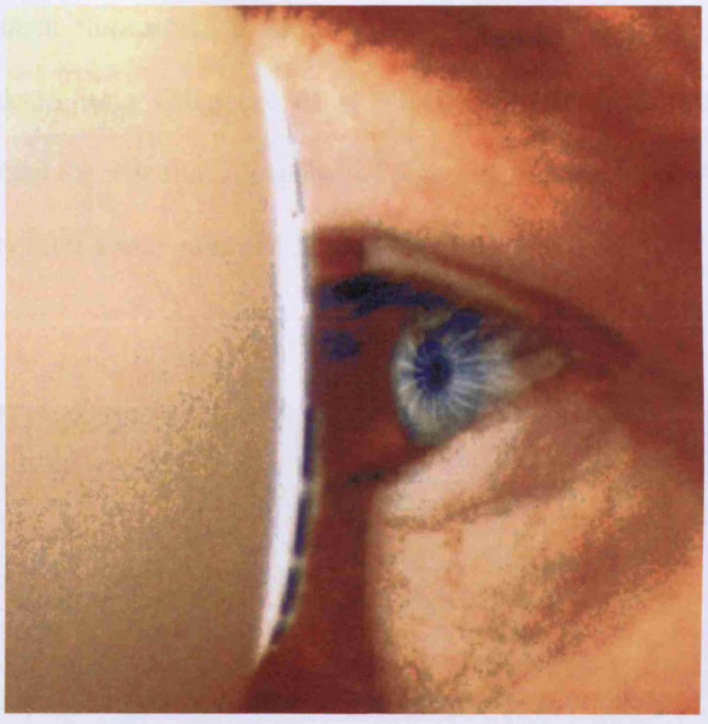


Figure 1.6 Measurement of NIBUT by the Tearscope (illustrated in Korb et al. (2002))

To improve the observation of tear break-up, fluorescein sodium stain is instilled. (Mengher et al., 1985b). When illuminated with a cobalt blue light from the biomicroscope and viewed through a yellow filter, the fluorescein has a yellow-green appearance in the tears. In contrast, breaks show as black areas within the stain. However, if too much fluorescein is used it will change the tear composition and decrease the break-up time (Mengher et al., 1985a; Patel et al., 1985). After fluorescein instillation the tear film is capable of regenerating quickly and it returns to the original stability after a maximum of 20 minutes.

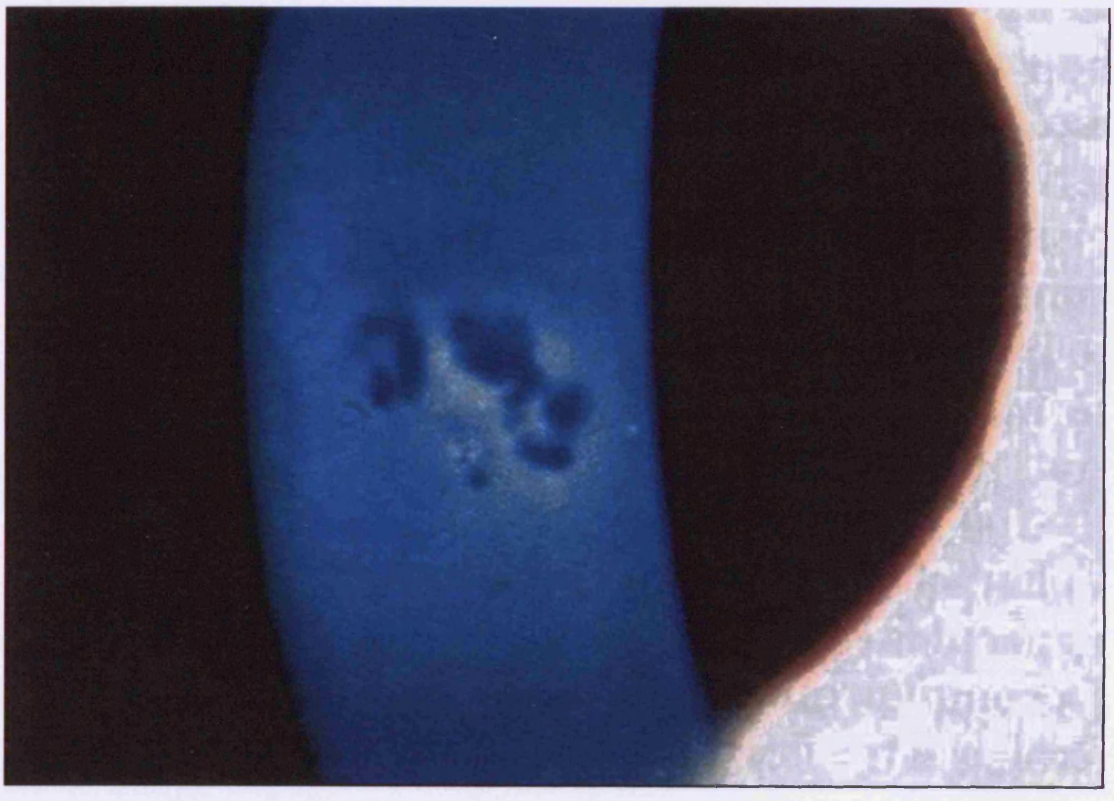


Figure 1.7 The tear film break up shows dark spots in its reflection and can be observed in the optic section of the slit-lamp illumination (illustrated in Efron (2002b)

1.2.8.5 Tear production and secretion tests

Quantitative tear measurements examine tear flow and tear secretion. Testing of these parameters is well-accepted for clinical use (Korb, 2000). All the presented tests in this section are easy to perform in a clinical setting, but rely on the observational skills of the examiner.

Tear flow is determined *in vivo* by viewing the fluorescein decay over time (Benedetto et al., 1984; Mathers and Daley, 1996). A more objective way is less clinical. The test can be performed by a measurement of fluorescein concentration, by collecting a tear sample after stain instillation. Two different ways of in *vitro* measurement are possible. Either the collection material, such as a Schirmer paper, can then be compared to photographic standards (Nava et al., 1997) or the concentration of the fluorescein in the tear sample can be measured optically (Afonso et al., 1999).

Tear collection by a Schirmer paper is only a side effect of its intended use. The standard Schirmer test is a measurement technique of tear production (Schirmer, 1903). A filter paper strip is bent at a notch close to one end and placed in the lower conjunctival sac (Figure 1.8). It is left for five minutes to soak tears from the tear meniscus (Yokoi et al., 2000) and the length of wet area is measured. If this area is below 5mm, the diagnosis is reduced tear secretion such as in dry eye (Korb et al., 2002). The Schirmer test can cause reflex tearing and serum leakage because of the mechanical irritation to the ocular surface (van Haeringen and Glasius, 1977; Clinch et al., 1983; Stuchell et al., 1984; Craig and Blades, 1999; Yokoi and Komuro, 2004). Thus, to measure tear volume, the Schirmer paper strip has been modified by

shortening the measurement time and by using local anaesthetics (Lamberts, Foster and Perry, 1979; Bawazeer and Hodge, 2003) or it can be replaced by the phenol red thread (Hamano et al., 1990; Mainstone, Bruce and Golding, 1996).

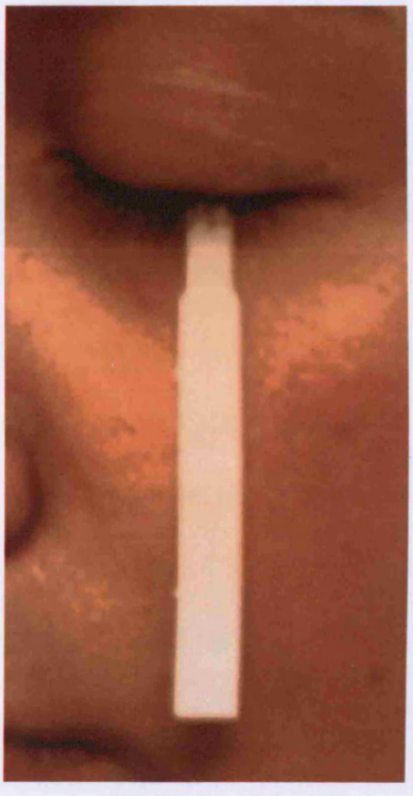


Figure 1.8 The Schirmer paper is placed on the lower lid. Eyes are closed during the test to prevent further stimulation.

Another useful assessment of the tear volume is to measure the tear prism or meniscus height. The tear fluid forms a meniscus shaped pool at the lids and is seen best on the lower lid. Usually the height and curvature are assessed by viewing the meniscus (Wright, 1971; Baum, 1973; Mainstone et al., 1996; Golding, Bruce and Mainstone, 1997). Newer investigative techniques for tear meniscus assessment employ

pachymeters (Port and Asaria, 1990), videography (Oguz, Yokoi and Kinoshita, 2000; Doughty, Laiquzzaman and Button, 2001) and optical coherence tomography (Johnson and Murphy, 2005).

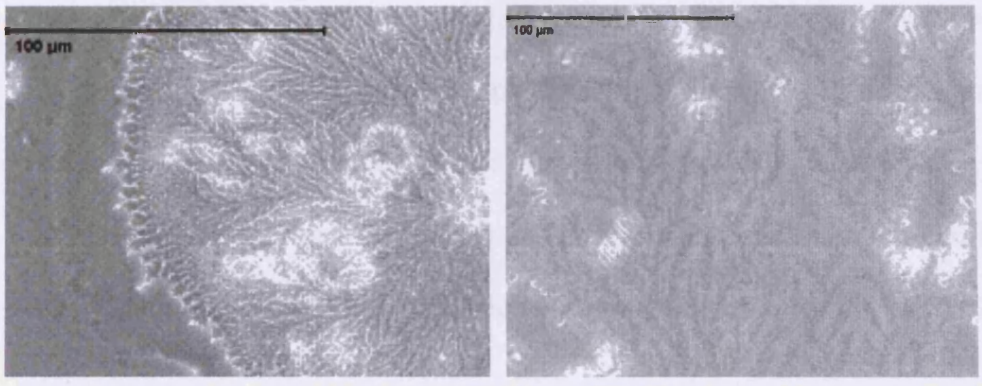
1.2.8.6 Tear fern ing test

Tear ferning is produced when a tear drop is dried on a glass plate, and shows a dendritic growth pattern similar to snow crystals. The fern pattern was originally used to examine the female menstrual cycle by testing the cervical mucous (Papanicolaou, 1946). However, it proved a useful technique to determine the equilibrium in the tear film. The characteristic pattern depends on electrolyte concentrations, the ratio between the mucous and the protein content, the degree of lipid contaminated mucous (in dry eyes), and any altered rheology of the tear film (Golding and Brennan, 1989). The crystallisation pattern varies under pathological conditions. A regular and dense tear ferning indicates a homeostatic and healthy tear film.

Different grading systems have been used for tear fern assessment (Rolando, 1984; Norn, 1987b; Pearce and Tomlinson, 2000). Tear ferns are generally graded according to Rolando (Rolando, 1984). He graded the regularity with type I, for the most uniform arborisation, type II for a pattern with gaps, type III for sporadic ferning, and type IV for when ferning is entirely absent (Figure 1.9). The tear film of healthy eyes have mainly type I and II grading. To improve the grading, Norn (1987b) proposed an equation that takes more factors into account such as magnification, density and ferning angles. A potential objective method for quantifying the fern pattern is to use spatial location coupled with scanning electron

microscopy and concurrent energy dispersive X-ray analysis (Golding et al., 1994; Pearce and Tomlinson, 2000).

a) Grade I b) Grade II



c) Grade III d) Grade IV

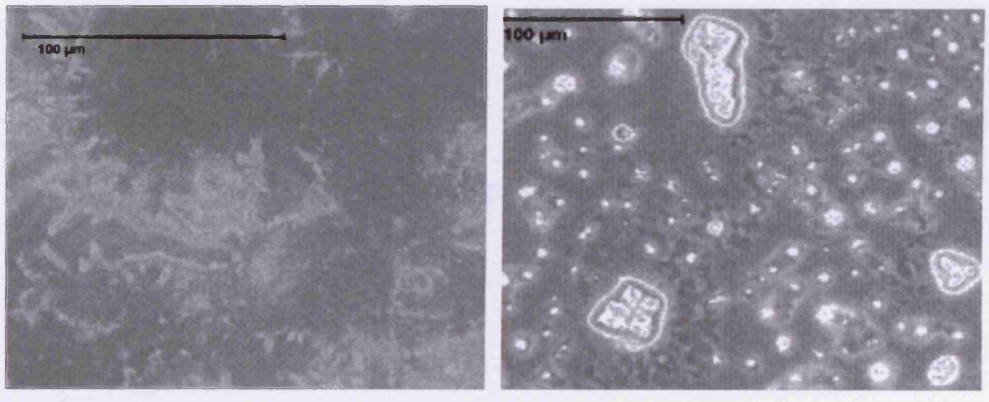


Figure 1.9 Example of tear ferning grading. Tears were collected from students and graded according to Rolando (Rolando, 1984): from a) grade I to d) grade IV with decreasing balance between all components

Rolando's grading system has been applied by other investigators. Ravazzoni et al. (Ravazzoni et al., 1998) tested the ferning method with subjects who were fitted for the first time with hydrophilic contact lenses and were followed up for 6months. They noticed a higher and earlier drop-out rate of contact lens wearers with higher grades of III and IV and recommended tear ferning as a predictive tool for contact lens tolerance. Other studies found that the quality of ferning in subjects with Down's syndrome and cystic fibrosis was lower than in subjects with no systemic diseases (Rolando, Baldi and Calabria, 1988; Filipello et al., 1992). Tear ferning is a clinical tool to diagnose dry eye disease (Norn, 1988; Vaikoussis, Georgiou and Nomicarios, 1994) and even to investigate neonate tear film (Puderbach and Stolze, 1991).

The application and observation of tear ferning is simple, rapid and of low cost. Its non-invasive integrity is dependent on the tear collection method used, but ferning doesn't require any use of topical anaesthesia. Another advantage is its reported reproducibility (Norn, 1994; Pensyl and Dillehay, 1998). In contrast to other clinical tests, its outcome is independent of provoked reflex tearing (Norn, 1988).

1.2.9 Biochemical analysis

Biochemical assays analyse a wide range of components and their functions, such as lipids, mucous, hormones, and nucleic acids. The biochemical analysis in this work focussed on proteins as they are essential components of the tear fluid (Whikehart, 2003).

Proteins are produced by genetic expression, and are responsible for many different qualities of the tear film. Proteins are composed of amino acids that are attached by

peptide bonds (Stryer, 1999). On the ocular surface and the tear film they have many roles, such as maintaining structure and clarity of the cornea by enzymatic processes, binding of minerals, lysis of bacteria, and other immune responses (Whikehart, 2003). Proteins are separated and visualised by different biochemical assays. These assays are based on the principles of chromatography.

1.2.9.1 Chromatography

Chromatography is used to detect proteins and determine their quantity. A crude way to determine the presence of a protein in a solution is by a simple UV detector. All proteins absorb UV light and measurement can be carried out at the absorption level of peptide bonds (Johnson, 1994). To enable specific quantification of proteins, a protein sensitive dye can be used that alters its optical density according to the concentration of proteins. The general term for this application is colourimetric assay. Many different colourimetric assays for determining total protein concentration in tears are available commercially, such as tests adapted from Bradford (Bradford, 1976) or Lowry (Lowry et al., 1951).

All available protein assays use the same principle for quantification. The measurement of the protein content has to be compared to a standard with known concentration. The chosen standard needs to ideally be the same protein as the unknown one. Not all human proteins are available as standards and sometimes a protein with a similar location and colour yield from a different host such as bovine or chicken is used. Typical standard proteins for routine assays are chicken egg lysozyme or bovine serum albumin. The standard is assayed in different dilutions similar to the expected concentration of the unknown protein and a standard curve is

plotted. Additionally the standard can give information about linearity and accuracy of the measurement.

As described earlier the tear film contains various proteins. For analysing their profile the proteins need to be separated in a controlled technique. To separate molecules based on differences in their structure or composition, the sample is moved over a stationary support. The molecules that have different interactions with the moving force will separate and elute differently. Once the different molecules are collected in different fractions, a detector can determine their concentration and chemical composition. The detector works, for example, on the principle of refractive index or UV absorbancy.

There are many different techniques and also a high amount of modifications and combinations of assays available. Generally the nature, meaning the sample concentration, determines the choice of technique. Other factors that determine the choice of assay are required precision, ease of use, safety and laboratory settings. A preferred method to examine the tear film profile is electrophoresis that uses electric power as the moving force.

1.2.9.2 Electrophoretic techniques

Electrophoretic techniques work on the basis that proteins are charged molecules and migrate in an electric field (Stryer, 1999). Different proteins form a profile and are located according to their migration rate. Different electrophoretic techniques exist, such as gel electrophoresis, immunoelectrophoresis and isoelectric focusing. These techniques use different ways to limit migration and enhance separation. For

example, they utilise mediums such as a gel with a specific pore size as in gel electrophoresis, an antibody within the gel as in immunoelectrophoresis or a continuous pH gradient such as in isoelectric focussing.

For human tears, gel electrophoresis has been recommended due to the high resolution and the clear differences between the profiles (Janssen and Van Bijsterveld, 1981; Berta, 1982). Gel electrophoresis exists in two types of applications (Hames and Rickwood, 1990). A one-dimensional gel electrophoresis spreads the sample in one direction. A two-dimensional extends separation by isoelectric focussing in a second direction and has a higher resolving power (Glasson et al., 1998). Due to cost and ease, the one dimensional application is the preferred method of use in many tear film studies (Tseng, Huang and Sutter, 1987; Bjerrum, Halken and Prause, 1991; Kuizenga et al., 1996). It offers the possibility of processing multiple samples on the same application.

1.2.9.3 Identification and quantification of a single protein

Within a protein profile, the identification of a protein of interest needs to be verified. For this purpose different methods were developed, such as Western blotting and enzyme-linked immunosorbent assay (ELISA).

Samples subjected to Western blotting are already separated by gel electrophoresis. After undergoing gel electrophoresis, the separated proteins are transferred from the gel to a thin support matrix that immobilises the protein (Hames and Rickwood, 1990). Primary and labelled secondary antibodies visualise the protein, which is semi-quantified using laser scanning densitometry. For quantification, Western

blotting is not as sensitive as the ELISA technique. ELISA's are used in routine screening procedures or to precisely measure the concentration of a given sample. An antigen- or antibody-coated plate is used to capture the antibody or antigen, respectively, in the sample. Another antigen or antibody, then binds to the sample and this agent can be labelled or be detected by a labelled reagent (Crowther, 2001). The label may be fluorescence, peroxidase or alkaline and will visualise in different colours. Similar to Western blotting, the proteins in a tear sample for the ELISA technique, can be first separated and purified by chromatography (Fullard, 1988). This process requires a bigger tear sample volume and more equipment. In contrast, Western blotting is easy to handle and is more cost effective when only a small number of samples are used each time. In addition a single blot can be used several times (Towbin and Gordon, 1984; Hames and Rickwood, 1990).

1.3 THE INFANT TEAR FILM

1.3.1 General development

An infant's state of growth is determined by their gestational age and birth weight. Infants born between the 38th and 42nd week of gestational age are full term infants, whereas all infants born earlier are referred to as preterm or premature born infants; if classified by weight, infants with less than 2500 g weight at birth are low birth weight infants and usually premature infants also have low birth weights (Osofsky, 1979; Snow, 1998).

In the first two years of life, motor activity and cognitive processes rely on sensory perception (Mandler, 19901992). That means that physiological sensory mechanisms start developing very early before birth. The sensory system to tactile stimuli is the first to develop at the 7th to 14th week of gestation (Gottfried, 1984). The hearing apparatus is complete at 20-24 week of gestation, but compared to an adult's ability, the infant's hearing is impaired at birth and shows high variability (Snow, 1998). Taste cells exist at 14th week of gestation and infants are born with only a sense for bitter sour and sweet, and a preference for the latter. Finally, the neurological pathways for pain perception are developed by the 29th week of gestation (Walco, Cassidy and Schechter, 1994) .

Although the physiological and neurological basis for sensation develops during the foetal stage, it continues to develop after birth. During this time the development is susceptible to changes. The time when these functions can be altered is called the critical period, and refers to when the infant is most open or vulnerable to

environmental influences (Snow, 1998). During this time intervention for an impairment is most effective to the infant. Vision is an important sense which must be tested early in infants and children to avoid the development of vision loss

1.3.2 The infant eye

1.3.2.1 Physiologic development of vision function

The development of vision is associated with neural development (Born et al., 2000). The pupil's reaction to light has been reported to develop in premature and full-term infants with maturation (Isenberg, Dang and Jotterand, 1989) and be measurable after the 31st week of gestational age. Similarly, neurological changes in the retina occur as part of the maturation. Foveal cones start to develop before birth. Cones for short, long and medium colour wavelengths, abbreviated S and L/M cones, appear between the 11th and 15th week of gestational age and spread until birth (Cornish et al., 2004). They continue to migrate centrally to form the foveola until an adult density is reached by around four years of age (Yuodelis and Hendrickson, 1986). If an infant's or child's stimulus for vision is deprived by a squint, refractive error or cataract, then visual acuity and/or binocular vision may be lost. The critical period when vision can be deprived (Wiesel and Hubel, 1963), is different for each visual function and generally falls between the onset of development and puberty (Daw, 1998).

Decreased vision is the main effect of deprivation in one or both eyes, and is called amblyopia (Hillis, 1986). It can be best improved with early intervention. It is generally an accepted practise that the improvement of clear vision has a priority in comparison to other functions (Daw, 1998).

At birth the visual system is not complete, but visual acuity measurements by behavioural (Fantz, 1965; Fantz and Fagan, 1975; Teller and Boothe, 1979; Atkinson, 1984) and electrophysiological (Norcia and Tyler, 1985; Sokol and Moskowitz, 1985; Hamer et al., 1989) methods show a rapid development within the first year of life. Similarly, other functions such as saccadic and pursuit eye movements, co-ordinated head and eye movement and vergence develop within the first six months of life (Moore, 1997). The reports of physiological and sensory development show that overall full maturation of the visual system may take longer depending on the visual function.

In contrast, premature infants can have a different development. Premature infants with a low birth weight may acquire retinopathy of prematurity (ROP). ROP is a retinal vascular disease (Terry, 1942; Patz, 1984) that manifests on the retina and has many effects on vision (McCormick et al., 1992; Robinson and O'Keefe, 1993). However, it was shown that even prematurely born children with no ROP and neurological disorders are at risk for visual impairment (Larsson, Rydberg and Holmstrom, 2005), reduced contrast sensitivity (Larsson, Rydberg and Holmstrom, 2006) and altered visual fields (Larsson, Martin and Holmstrom, 2004).

1.3.2.2 Vision correction for infants

The earliest vision correction is usually attempted for aphakic infants that have undergone surgical lens extraction for cataracts. In addition to congenital cataracts, some children and infants with high refractive errors or anisometropias are fitted with contact lenses. For amblyopia treatment, contact lenses have been shown to

overcome the major problem of compliance (Roberts and Adams, 2002). Infants are fitted with soft hydrogel or silicone hydrogel extended wear lenses (Martin et al., 1983; Neumann et al., 1993; Ozbek, Durak and Berk, 2002) . Due to high plus thickness and a consequent reduction in oxygen permeability, rigid gas permeable (RGP) lenses are also recommended and have been reported to be easier, more cost effective, and to offer a good correction for astigmatism in infants (Amos, Lambert and Ward, 1992). However, Neumann et al. (1993) found that one of the main complications of long term contact lens wear in infants and children is infections. De Brabander et al. (de Brabander et al., 2002) described difficulties in paediatric contact lens wear mainly to be due to deposits, more often from the age of three years. In adults, contact lens deposits are due to tear lipids and proteins (Wedler, 1977; Hart, Tidsale and Sack, 1986; Sack et al., 1987; Keith et al., 2003).

1.3.3 Infant tears

Previous studies involving infant tears were conducted in conjunction with general ophthalmologic practice. These studies examined the effects of drugs on the ocular surface and how they are affected by dilution or absence of tears; or on the effect of diseases on tear components. For example, ophthalmic medications used on the eye surface were proven to have systemic effects on infants. Mydriatics such as phenylepherine hydrochloride above 2.5% raise the systolic blood pressure (Isenberg and Everett, 1984) and cyclopentolate hydrochloride raises gastric acid secretion (Isenberg, Abrams and Hyman, 1985). These effects were reported in low birth-weight neonates and premature infants.

Ophthalmological interest has also been focussed on a tearing disorder that is very common in young infants. It is called epiphora and affects up to 20% of infants during their first year of life (MacEwen and Young, 1991). In these infants the tear secretion is higher than the nasolacrimal drainage is capable of disposing. Usually no intervention is needed and the epiphora resolves on its own. If not, the drainage can be opened by probing or surgery (Stevens, 1998; Marr, Drake-Lee and Willshaw, 2005). Probing is also the first procedure when congenital nasolacrimal duct obstruction leads to an inflammation, such as acute dacryocystitis (Greenberg and Pollard, 2003). Inflammations of the nasolacrimal ducts and bacterial inflammation of the conjunctiva can also be cured by antibiotics (Friendly, 1983).

Recently, improved contact lens materials, more frequent contact lens replacement and the challenge of disorders of the tears themselves (such as dry eye syndrome) have provoked a lot of specific research and development of sensitive methods to examine every aspect of tears. However, these examinations have not been sufficiently applied on infant tears and the amount of knowledge on normal tear secretion and its development in infants is scarce.

1.3.4 Components of the infant tear film

1.3.4.1 Mucous

As described earlier, the tear film component covering the ocular surface is the mucous layer. The equilibrium of the different constituents in the mucin gel can be determined using the tear ferning technique (Golding and Brennan, 1989), which involves observing the fern like pattern of a dried drop of tears on a glass plate. A

grading of the observed pattern from type I, for the most uniform arborisation to type IV, when ferning is entirely absent (1984), demonstrates that the tear film of normal, adult eyes is mainly type I and II. Puderbach and Stolze (1991) used the technique of tear ferning on a group of children with an age range of four months to ten years. They found that 17% had type I and 63% type II ferning, according to Rolando's grading system (1984), which was similar to non-symptomatic, normal, adult eyes. Norn (1988) took scrapings from infants with neonatal conjunctivitis and found similar types of fern pattern as in other bacterial infections with presenting with high mucous content.

In contrast to what is known about the full-term neonate, there is a limited amount known about the tear composition of premature infants. However, the epithelial layer of the ocular surface is underdeveloped (Dogru et al., 2004), which may cause a deficient mucous composition.

1.3.4.2 Aqueous in infant tears

Historically an immature nervous system in neonates was assumed. This led to the idea of a delayed development of the lacrimal gland and delayed aqueous secretion. Basal and reflex tears were thought to be absent until a few weeks after birth (Mutch, 1944), although it was known that the capacity for normal lacrimation existed at birth anatomically (Axenfeld, 1898).

The Schirmer test was used to find the onset of tear secretion, the secretion rate and its maturation in normal and premature infants (Sjogren, 1955; Apt and Cullen, 1964; Patrick, 1974; Spiegler and Mayer, 1993; Isenberg et al., 1998; Menon, 2000; Toker

et al., 2002; Akar et al., 2004; Dogru et al., 2004; Rohatgi et al., 2005). This method had two disadvantages. Firstly, topical anaesthesia was required in order to measure the basal tear rate. Secondly, the eyes of the infants proved too small for the strip, so that contact with the cornea could not be avoided (Apt and Cullen, 1964).

However, these studies demonstrated that neonates have both basal and reflex tearing soon after birth, although the production rate is not at adult levels. Penbharkkul and Karelitz (1962) investigated lacrimation by awaiting or provoking crying in neonates. They observed the earliest example of the first shedding of tears at five and a half hours after birth and the latest by the 60th day, postpartum. Sjögren (1955) observed reflex tearing in 13% of full-term newborns at birth, with 35% of these having a normal secretion rate. Patrick (1974) found most infants tearing normally when tested with the Schirmer test and reported that, at two days of age, 87% of neonates exhibited some tear secretion, increasing to 95% after the first week of life. Apt and Cullen (1964) reported a high incidence of normal tear secretion on the first day of life in 96% of neonates assessed.

This variation in basal tear secretion has no correlation with birth weight or maturity in full term neonates, and there is no general relationship between basal tear secretion and gender or maturation (Patrick, 1974; Spiegler and Mayer, 1993; Menon, 2000; Toker et al., 2002; Akar et al., 2004). The findings on basal and reflex tear secretion illustrate that, although the data found for both basal and reflex secretion in neonates is generally less than in adults, it is still above the suggested threshold for dry eye (5mm Schirmer wetting with anaesthesia and 15mm without) and can therefore be regarded as normal. Presumably the secretion rates reach higher adult-like values

when the lacrimal gland achieves full anatomical development at three to four years of age (Isenberg, 1989).

1.3.4.3 Proteins in infants

In the same way as the adult tear film, the neonate tear film contains a wide range of proteins. Since the identification of proteins can be used to determine the source and origin of tear flow, protein assays are useful in understanding the changes in the anatomy and structure of the lacrimal gland after birth.

Lysozyme is one of the main lacrimal gland proteins. It is a regulated protein (Dartt, 1989) meaning that its rate of secretion is constant at all flow rates (Fullard and Tucker, 1991) and that the effects of tear stimulation cannot diminish the presence of lysozyme. Etches et al. (1979) reported a measured value of 1.12 μg/μl in a group of infants which is lower compared to the lysozyme concentration in adults of 1.7 μg/μl (Fullard and Snyder, 1990). Etches et al's value is higher than that previously reported by Bonavida and Sapse (1969), who analysed the lysozyme proteins of just four infants and found a concentration of 0.7-0.8 μg/μl.

Allerhand et al. (1963) examined the tears of neonates, who cried tears within 36 hours of birth. They were the first to show the existence of lysozyme electrophoretically and immunochemically in neonatal tears. In addition to lysozyme, proteins similar to those of serum, particularly alpha-1 globulin was found. In contrast, older infants lacked alpha-1 and showed alpha-2 trace. Allerhand et al. (1963) also suggested that the total protein content in neonates is greater. They demonstrated the protein content by paper electrophoresis and showed the major

proteins albumin, lysozyme, and middle fractions at comparable levels to that of adults (Apt and Cullen, 1964).

The lacrimal gland appears to be the only source of secretory IgA. IgA was found to exist at concentrations ranging from 2% to 7% of normal adult levels (Patrick, 1974). In normal infants and children, the concentration of the free secretory component tends to be lower than in adults. The secretory component, the IgA and lysozyme in mucosal surfaces, such as the ocular surface, are vulnerable defensive agents in infants and children and their levels can be decreased by malnutrition. In that case, the reduction of IgA will be more severe in children than adults. (Cheatham and Michalek, 1984). In a study by McMurray et al. (1977), IgG levels of older infants between 1.5 to 2 years of age were found to be reduced by malnutrition. Total protein, serum albumin, lysozyme, and aminopeptidase, a catalytic enzyme to process peptide bonds (Calderon de la Barca Gazquez et al., 1989), were unaffected.

1.3.4.4 Lipids in infants

The neonate tear film has an unusually high stability due to a thick lipid layer (Isenberg et al., 2003; Lawrenson et al., 2005). Interference patterns associated with a thick lipid layer were found with a gradual thinning of the layer towards adult levels by one year of age (Lawrenson, Murphy and Esmaeelpour, 2003). It was concluded that this thick lipid layer stability reduces the need for frequent blinking. In another study, Isenberg et al. (Isenberg et al., 2003) measured the lipid layer thickness and tear film break-up times in preterm and full-term neonates followed up to the age of six months. They found the thickness to be significantly higher than other studies reported for adults. As the evaporation rate is decreased by a thick lipid layer, the

results of tear film break-up times also exceeded results for adults. Similar results were found by Kaercher et al. (1994), when compressibility and surface tensions of the meibomian gland secretions from subjects (0-1.5 years, 4-5 years and 7-10 years of age) were measured *in vitro*. Infants in the 1.5 years group demonstrated a superior surface tension, much better than adult meibomian gland secretions. There are two theories explaining the relationship between the lipid layer and the tear stability in infants. The first theory is that the quality of infant tear lipids might differ to adult's lipids. This was shown for the meibomian lipids, such as a lower level of free fatty acids in infant tears (Shine et al., 2004). A second theory is that the smaller eye surface of infants compresses the amount of lipids so that the thickness is not due to the amount of secreted lipids (Isenberg et al., 2003; Lawrenson et al., 2005).

1.3.5 Blink mechanism in infants

As with the neonate tear film the earliest investigations of the infant blink mechanism were influenced by concurrent theories on maturation of the nervous system. Ponder and Kennedy (1928) observed low blink rates which led them to propose that normal blinking did not develop until the age of six months. They linked any observed blinks before that time as being caused by some external stimulus, such as an unexpected sound or movement.

Neurological development could either be delayed at the corneal nerve site, or delayed in the higher brain centres that control blinking. To investigate the neurological development and its effect on the blink rate, the corneal nerves were investigated for their tactile sensitivity. Dogru et al. (Dogru et al., 2004) used a Cochet-Bonnet aesthesiometer to assess corneal sensitivity in premature and full-term infants. A thin

fibre was pressed against the cornea until a blink reflex was evoked. The length of the fibre indicated the sensitivity in which a short fibre below 50mm in length indicated a decreased sensitivity (Martin and Safran, 1988). They found a low sensitivity in 87.5% of the premature infants and a statistical difference with the full-term group. In support of the underdeveloped corneal nerves theory, Snir et al. (2002) reported that the tactile corneal reflex was not commonly present in babies during the first week of life, although it gradually increased to normal levels by the 12^{th} week.

In contrast, Lawrenson et al. (Lawrenson et al., 2003) found no significant difference in sensitivity between neonates and adults. They assessed corneal sensitivity using a cooling stimulus that stimulated the C-fibres. Their results suggest that a well-developed thermal sensitivity is present at birth. Lawrenson et al. (Lawrenson et al., 2003) also found blink rates in newborns typically between 2 and 3 blinks per minute, well below the adult blink rate of 12 to 20 blinks per minute.

1.4 TEAR COLLECTION

A variety of techniques have been employed to collect a suitable tear sample (Stuchell et al., 1984; Webster and Kairys, 1984; Jones, Monroy and Pflugfelder, 1997; Dumortier and Chaumeil, 2004; Lopez-Cisternas et al., 2006). Tear collection methods vary according to the amount of tears required. Sample size and collection technique may irritate the ocular surface and alter tear composition (van Haeringen and Glasius, 1976). Considering the difficulties with reflex tearing, the sample size should be kept small. Fullard and Snyder (Fullard and Snyder, 1990) were able to take a 10µl tear sample without any stimulation over a period of 24 minutes. They used glass capillary tubes and collected carefully in 1.5µl steps in order not to exceed a collection rate of 0.5µl/min. However, when cooperation is needed from the patient, this tear collection method is not suitable.

1.4.1 Collection techniques

Glass capillary micropipettes are suggested to be the gold standard, but they have their limitations. They collect tears slowly and unpredictably, and are cumbersome to use in a clinical setting (Jones et al., 1997). Collection of tears by means of capillaries demands co-operation from the subject. Therefore this method is not useful for mass screening, especially if children are investigated (van Agtmaal et al., 1987; Norn, 1992).

Some alternative invasive collection methods require the use of anaesthetics. For example, spatulas, collection papers and threads, and sponges. Some methods such as Schirmer papers and sponges utilise absorbent materials atraumatically but with

ocular surface irritation. Spatulas are the most invasive, if used to take conjunctival mucin by scraping the lower nasal palpebral conjunctiva (Tabbara and Okumoto, 1982). Analysis of serum albumin may suggest the presence of undesirable irritation and is a useful control measure for provoked exudation (Norn, 1992). Some collection methods, such as porous polyester rods, glass rods, and loops, attempt to dip just the surface of the tear film without touching the ocular tissues. Similar to sponges, porous polyester rods are absorbent materials and, compared to glass capillary micropipettes, the rods are 3.9-fold faster in collecting tears and equally efficient in the recovery of proteins (Jones et al., 1997).

1.4.2 Stimulation

If proteins have to be analysed, the amount of required tears depends on which protein is targeted. Collected tear volume above the normal tear flow can be considered as stimulated. Knowledge of the effect on tear components by mechanical irritation or stimulation could lead to a better assessment of test results for proteins (Fullard and Snyder, 1990; Fullard and Tucker, 1991). If several tear fluid tests have to be undertaken, the order of tear samplings should consider the effects of stimulation. Even though certain components such as the solute weight (Farris et al., 1981) or serum albumin (Prause, 1983) vary with tear fluid levels, the ratio balance between them seems to be the least influenced by reflex tear flow (Norn, 1988).

1.4.3 Sample treatment

1.4.3.1 Fluid recovery

Once the tears are collected they need to be expelled and prepared for further analysis. Tear fluid recovery from absorbent materials can be performed in different ways. Using Schirmer papers, the tears are obtained after incubation and elution of the material (Puderbach and Stolze, 1991) to extract proteins. If the tear fluid is expressed by compressing sponges (McMurray et al., 1977; Watson et al., 1985), some of the sample will remain inside. Tear fluid is recovered from sponges by centrifugation (van Agtmaal et al., 1987). The absorption effects of cellulose sponges seem to be 6 times more than that for filter papers (van Agtmaal et al., 1987), which might reduce the effects of evaporation (Tuft and Dart, 1989), so that cellulose sponges lose less to evaporation.

1.4.3.2 Centrifugation

Centrifugation is a well controlled method for the purpose of sample recovery. Optimal spinning duration and force for gaining the most amount of fluid has to be determined according to the sample. Analysis of proteins has shown that a long centrifugation time and filtering of the sample leads to a small concentrated protein volume, and the protein bands obtained after concentration become more distinct (Bjerrum and Prause, 1994).

1.4.3.3 Elution

Another method used to retrieve tear fluid and proteins from an absorbent material is elution (Watson et al., 1985). The material is left in buffer before centrifugation. The

buffer disrupts the protein binding to the material by changing the environment, e.g., the pH or the protein structure. Additionally the sample is diluted and this needs to be considered when analysing the concentration. After sufficient incubation, the material is centrifuged to obtain the complete sample and added buffer.

1.4.4 Dilution of Samples

To minimise effects of evaporation in small samples, the recovered tear fluid can be diluted by distilled water or buffer. Bjerrum and Prause (1994) used a dilution rate of 1:3 to prepare their samples for storage. One drawback is that if the sample is very small the dilution might offer a bigger surface for the contents to attach to the sample container.

1.4.5 Storage of Samples

Often samples cannot be processed immediately after collection, they might need to be transported, or the remaining sample after an analysis assay needs to be stored. Sitaramamma et al. (1998a) determined the effect of storage on total tear protein and major protein concentrations. They used tear samples collected with a capillary tube and analysed the tears with SDS-PAGE, Bradford's method and HPLC. No change in total protein concentration occurred when tear samples were stored at room temperature for four hours for closed eye tears, or eight hours for basal and stimulated tears. Longer storage of closed eye and reflex tears at 4°C, –20°C, and –70°C is possible for one week, up to two months, and up to four months, respectively. However, freezing at –20°C and –70°C for 4 months reduces the amount of secretory IgA in closed eye tears and lysozyme in reflex tears.

In an investigation with an absorbent tear collection material, Jones et al. (Jones et al., 1997) stored polyester rods after tear sampling at –70°C for various days in a storage tube that did not contain proteinase inhibitors. They found a decrease of lactoferrin concentrations and detected an increase of EGF in a single sample after 2-5 days.

1.5 Summary of review

The literature review has established that the tear film is important for ocular health and clear vision. The tear film builds a barrier before the ocular surface and protects it from many different invaders such as bacteria and viruses. It can trap foreign bodies and wash them away, with the assistance of the blink mechanism. The tear film prevents the ocular surface from drying and provides the environment and nutrition necessary for the superficial cells to survive. Additionally it smoothes the surface and is important for the optical quality of vision.

The tear film components are produced by different tissues, each of which has a different method of secretion control. The integrity of the tear film is easily observed by many clinical methods. It has been found that in subjects describing discomfort, the tear film has often lost its stability and some elements are altered. The subsequent diagnosis is some manifestation of the dry eye syndrome. To understand the difference between a healthy and a compromised tear film, the components in the tear film need to be identified and compared. Hence, tears need to be collected. This collection process is a challenging technical "mountain" to climb.

The current standard tear collection method is a glass capillary tube. Tear fluid is drawn into the tube from the tear meniscus by capillary action. It can then be expelled immediately and used for different analysis assays. However, the collected tears vary in composition. This can be due to a normal variation between subjects or from day to day. It can also happen when the capillary tube touches the eye lids and lashes. The alteration of the tear film is even more dramatic when damage is caused to the ocular surface. For this reason, in a research setting, the subject has to be trained. To

avoid reflex tearing, training can be difficult in a clinical setting, and especially with subjects who cannot control head and eye movements. Tear collection from neonates presents just such a challenge.

The neonatal tear film shows an unusual stability. This stability is associated with a less frequent blink rate of neonates. A thick lipid layer provides the high level of tear stability. The thickness may result in a slower tear drainage that could be important for the developing ocular surface to have enough time to receive nutrition. However, during early childhood the neonatal tear film develops gradually to adult levels.

This previous research is only a beginning. Present knowledge of neonatal tears arises from older studies with small sample sizes. However, it is a step forward, opening the opportunity to examine other aspects of neonatal tears. Some studies had low numbers of infant subjects and/or a wide age range. The principal reason for this can be attributed to difficulties in collecting tear samples and insufficient volumes for analysis. Several studies have used the Schirmer strip, usually to determine tear secretion, but topical anaesthesia was often required in order to measure the basal tear rate. Anaesthesia and prolonged invasive contact with the ocular surface can alter the flow rate by changing the sensitivity of the ocular surface (Jordan and Baum, 1980).

1.6 AIMS

To enable further studies on neonatal and infant tears, a new collection technique is necessary. It needs to quickly collect a tear sample without any damage to the eye. The use of absorbent materials is proposed: this technique should be less invasive and time consuming than the Schirmer strip, take up and release high tear volumes and be of less hazard to the ocular surface than the glass capillary tube. The next chapters lead step by step towards finding and using this alternative method. Therefore the aims of each chapter is summarised by the following questions:

Which methods and materials are available for tear collection (Chapter 2)?

To select the most suitable material and to evaluate the optimum of extraction method, the following aims were established:

- To select potential absorbent materials for tear collection.
- To examine the properties of fluid extraction.
- To choose an appropriate material that does not affect the analysis assays.

How suitable is tear sample collection with the selected method (Chapter 4)?

To compare this new method to the standard collection technique, the aims were:

- To show efficiency of tear collection with each collection technique.
- To investigate storage within the selected material.
- To assay protein content for both techniques.
- To assess the safety of the technique for the ocular surface.

How useful is the new collection method to investigate infant tear film (Chapter 5)?

To assess its efficiency for premature and newborn full-term infants, the main aims of this part of the work were:

- To evaluate the collection methods utility on an infant population.
- To apply the collection method to various sub-groups of infants: premature infants, full-term newborns and older full-term infants, in order to investigate differences in collected tear volume and protein content between sub-groups.
- To investigate the variables affecting the infant tear film.

Can the technique be extended to assessing clinical problems with paediatric contact lens fitting (Chapter 6)?

To apply the collection method to a practical clinical situation, this preliminary study had the following aims:

- To assess the process of tear collection and sample transport to the analysis facility
- To examine the efficiency and variability of the tear film in contact lens wearers from a wider age range

2 Establishing the optimum tear collection material and sample extraction method

2.1 INTRODUCTION AND AIMS

The first step in developing an alternative tear collection technique is the selection of the optimum material. Other investigators, such as Jones et al., defined the ideal collection method (Jones et al., 1997; Jones et al., 1998) and concluded that it should

- permit rapid collection with no ocular irritation,
- allow efficient and reproducible tear recovery,
- be applicable to sensitive quantitative analysis, and
- be readily applied to a clinical setting

This chapter reports on the development of new tear collection techniques that are proposed as an alternative to the glass capillary tube: absorbent materials that collect tears non-invasively. A variety of potential materials were selected and in-*vitro* modelling was conducted to assess their collection and extraction properties. In addition, the sample extraction method was optimised and the influence of tear storage assessed. Finally, the materials in question were tested to assess any effect they may have on the assays for which their samples will be used. These experiments had the following aims:

- To determine which test material is best for absorption and extraction (Experiment 1) and how centrifugation affects different tear-mimicking solutions (Experiment 2)

- To determine the optimal centrifugation operating modes (Experiment 3)
- To determine the effect of delayed processing caused by storage and freezing (Experiment 4) and the effect of sterilisation by UV radiation (Experiment 5)
- To determine the utility of the collection material for protein assays (Experiment 6)

The aims were examined in separate experiments. In the first experiment, the chosen materials were examined for their extraction properties. The effect of the solution property was examined by comparing solutions with different viscosities. Once the ideal materials were identified, the two materials with the highest extraction rate were used for testing the effect of centrifuge parameters spin duration, force and temperature, storage, and sterilisation.

For each experiment the samples were prepared and measured in the same way. The next section will explain the preparation of each sample.

2.1.1 Sample preparation

Each sample was treated the same way. Each material was cut and modified until the most practical shape and thickness was obtained for handling with forceps. The size was adjusted to make sure each piece could absorb a minimum of 30µl. This was done by dipping the material (in its ideal size and shape) into distilled water. Once full absorption was observed the fluid was extracted and measured as explained in detail later in this section.

For each experiment six preparations were made. For test purposes, the number of materials was reduced to six. First the solution was taken up by a pipette 0.5-10 µl (Biohit, UK). The test material was held with forceps and the pipette tip was placed at the surface that would face the tear meniscus. The solution was inserted into the material. This took less than a second, although it was observed that some materials absorb fluid faster (both kind of rods) and some are slow and change their shape after absorption (both kind of spears).

The material was immediately placed into a centrifuge tube that could contain a maximum volume of 0.2ml fluid (Figure 2.1). This centrifuge tube was closed on the top and a length of about 0.5mm was cut from the end tip to create a hole of approximately 1mm diameter. This tube was placed into a bigger tube (0.5ml void volume). The top of this larger tube was sealed with parafilm including the lid in order to protecting it from being damaged by the spin force. All tubes were placed into an adapter to securely fit the centrifuge inner ring holes and positioned on opposite sides for better balance. The sample was then processed in the centrifuge. Thus, the sample solution was extracted and collected in the larger tube. After centrifugation the smaller tube was disposed and the lid of the sample container was closed. It was then placed on ice ready for the next step.

Figure 2.1 Preparing polyester rod material samples for centrifugation

2.1.2 Measurement of extracted volume

After centrifugation, the sample volumes extracted needed to be measured. There is a risk of biasing the results by knowing which samples were extracted from the different materials. To avoid this bias, after centrifugation and before measuring the volume, the samples were rearranged by a co-worker. Each sample was labelled with a number. After rearranging, the co-worker wrote down the new order of numbers and erased the number from the lid of the tube. The written order was kept secret until all volumes were measured.

Once samples were extracted the volume was assessed. Measuring the weight did not prove sensitive enough to the small amount of volume differences. Instead two pipettes were used to soak the volume. These had ranges between 0.25-2.5 and 0.5-10 µl (Biohit, UK). The initial volume was easily estimated and the pipette was altered accordingly. The solution was taken up and expelled on a tissue that dried the pipette tip. Remaining solution was similarly estimated and taken up until no volume was left in the tube. Usually not more than two steps were necessary.

2.2 EXPERIMENT 1: Test material absorption and extraction qualities

The review of previous literature on tear collection suggested that polyester or cellulose based materials offered the greatest potential for efficient sample collection and extraction. This section will present chosen materials that need to be compared to each other.

2.2.1 Methods and materials

The process of centrifugation will be described in detail in the next experiment of centrifugation parameters. For this experiment a standard parameter of 10min spin duration, at the highest available rotation 15300 x g, at 4°C was chosen. Samples were prepared and used immediately. For aspiration into the materials, instead of using distilled water, a solution was chosen that resembled the viscose properties of the tear film.

Opti-Tears™ (Alcon Laboratories Inc, UK) are rewetting drops with a viscosity of 5-12 mPa.sec. Rewetting drops are indicated when a foreign body causing discomfort has to be removed. Additionally, the conditioning of contact lenses with rewetting drops can improve the comfort of contact lens wear (Coles et al., 2004). According to the supplier's information, this product contains Dextran (reference: instruction leaflet). Dextran is a polysaccharide with a molecular weight of 70kDAs (Norn and Opauszki, 1977; Murube, Paterson and Murube, 1998). This molecule is the main factor in determining viscosity.

To examine all test materials a standard volume of 10µl solution was applied to each test material with a micropipette (0.5-10µl, Biohit, UK). This chosen volume might be larger than tear volumes expected to be collected. However, this volume size was easy to measure and it reduced the effect of any uncontrollable factors such as evaporation.

By searching catalogues for ophthalmic surgery supplies and by contacting specialised manufacturers, a variety of materials were obtained. The following materials were selected for this study:

- Polyester rods

Polyester is a synthetic polymer (Josephy and Radt, 1940). Long chains are made by a reaction of an alcohol with an acid. The fibres in the rod are heat bonded and there is a void volume of 63% (Filtrona Fibertec, Germany). The polyester rod is used as an ink reservoir for writing instruments such as in markers. The rod was 3cm long, with a diameter of 0.21cm.

- Cellulose rods

The material is cellulose acetate. Cellulose consists of complex carbohydrates and is the basic structural component of plant cell walls (Josephy and Radt, 1940). It becomes cellulose acetate when treated with aceitic acid. The rod is used as ink reservoir in low cost writing instruments, suited for example for very large marker applications. The cellulose rod was supplied in a 2.5cm length and 0.2cm diameter (Filtrona Fibertec, Germany). Its void volume is 65%.

The cellulose rod differs from the polyester rod in its rigidity (it is softer) but not in pore size. Both rods had the same appearance and it is not easy to differentiate in any other way than by size or rigidity. The rods were prepared for the experiment by cutting them into pieces of approximately 1cm in length (Figure 2.2).

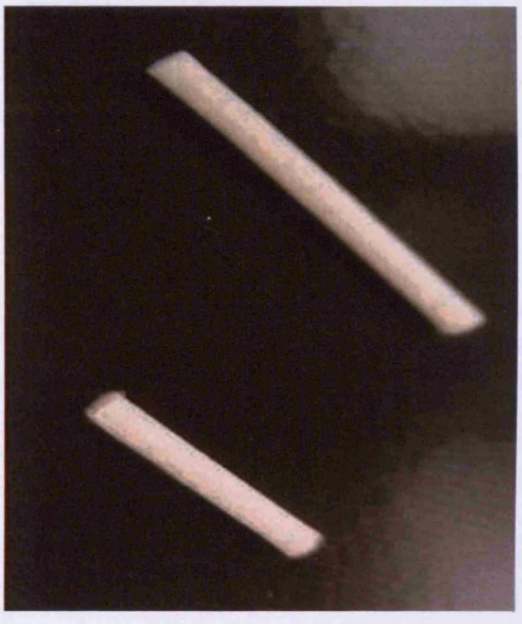


Figure 2.2 A cellulose rod in original size and cut to 1cm length (Filtrona Fibertec, Germany)

- PVA (or polyvinyl alcohol) spears

PVA spears (Altomed, UK) are single use products, used in eye surgery to collect blood and aqueous. They are supplied as spears and the tip was removed from the plastic shaft before centrifugation (Figure 2.3). According to the supplier's information the material is a polyvinyl formal resin and is hydrophilic. It absorbs 10-15 times its weight. The porosity is 90-92% and the pores are described as labyrinthal and continuous.

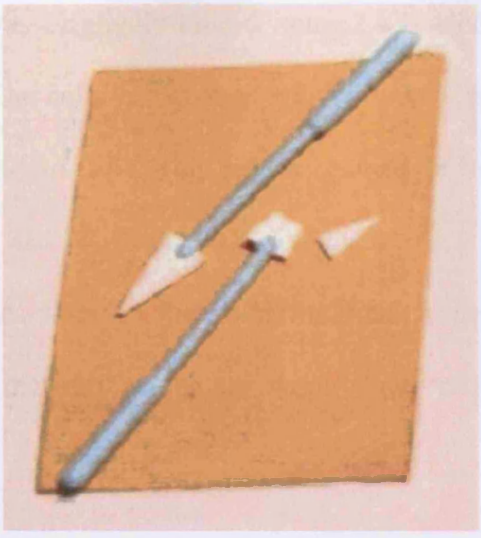


Figure 2.3 Manufactorer's supplied format of PVA and cellulose spears (Altomed, UK). The PVA material in this figure did not differ in its appearance from the cellulose material. The lower spear is cut to demonstrate the size and shape of material for assessment.

- Cellulose spears

Cellulose spears (Altomed, UK) are made of 100% regenerated cellulose. From a dry state, they absorb 15-17 times their weight. No information was available about the porosity and hydrophilic properties of this material.

- Cellulose sponge

Cellulose sponge is made from viscose rayon. Historically, viscose was produced from cellulose as a substitute for silk (Josephy and Radt, 1940). However, viscose is not a synthetic but a modified material from natural origin. According to the manufacturer, it absorbs nine times its weight in water, but is able to release 90% of

its liquid when pressed out. In the study, this material was referred to as a cellulose sponge. The cellulose sponge was supplied by Cotswold Outdoor, UK under the name PackTowel and was originally blue (Figure 2.4). Upon request, an unstained sample was supplied. The cellulose sponge was cut into long pieces of 5mm length and 2mm width for standard use. This length seemed appropriate, as it was a soft material and any bigger size was not easy to control. As is obvious from Figure 3, right image, the cut edges were not clearly defined and had tiny threads sticking out randomly. That means that any wider cut would have risked stimulating a larger surface area.

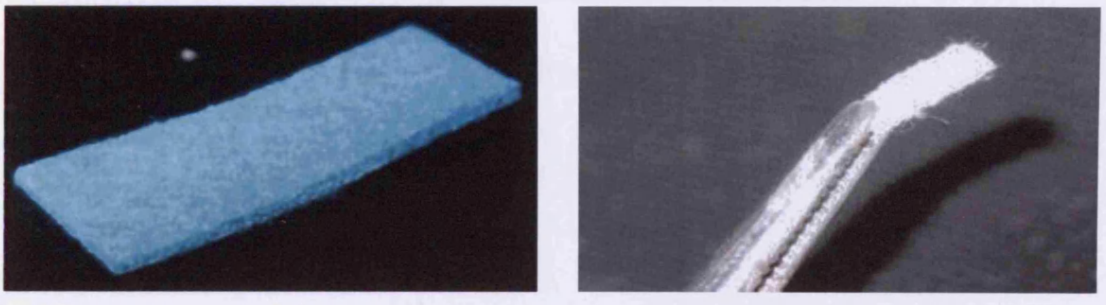


Figure 2.4 Manufacturer's supplied format of cellulose sponge (left) and an unstained format in the correct shape for usage (Cotswold Outdoor, UK)

- Schirmer paper strip

Schirmer strips are commonly used for the measurement of tear secretion rate. However, they have also been used for tear collection. The strips (Sno Strips, Chauvin Pharmaceuticals Ltd., UK) were cut into pieces of 1cm (Figure 2.5).

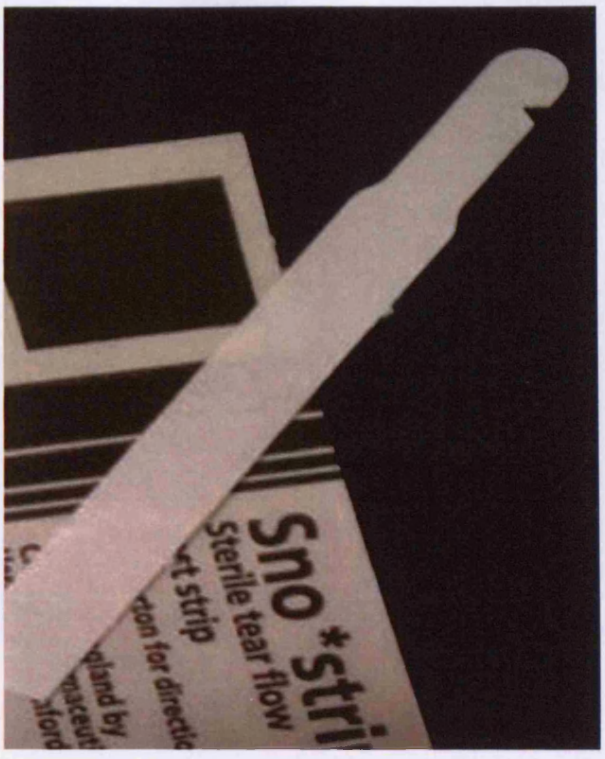


Figure 2.5 Schirmer paper strip

2.2.2 Results

Normality was checked by appropriate tests ($p>0.05$, One-Sample Kolmogorov-Smirnov Test) and observation methods (plots such as Q-Q plot of the residuals) were used to test the results for normality (SPSS11, USA). A one-way ANOVA test was used to examine the effect of the material on the extracted volumes. Overall the difference between the materials was significant ($p<0.001$).

Further statistics (Post hoc Tukey) revealed the difference between the materials. The difference between polyester rod, cellulose rod and cellulose sponge was not significant (each $p>0.05$). Figure 2.6 presents extraction volume results with each material.

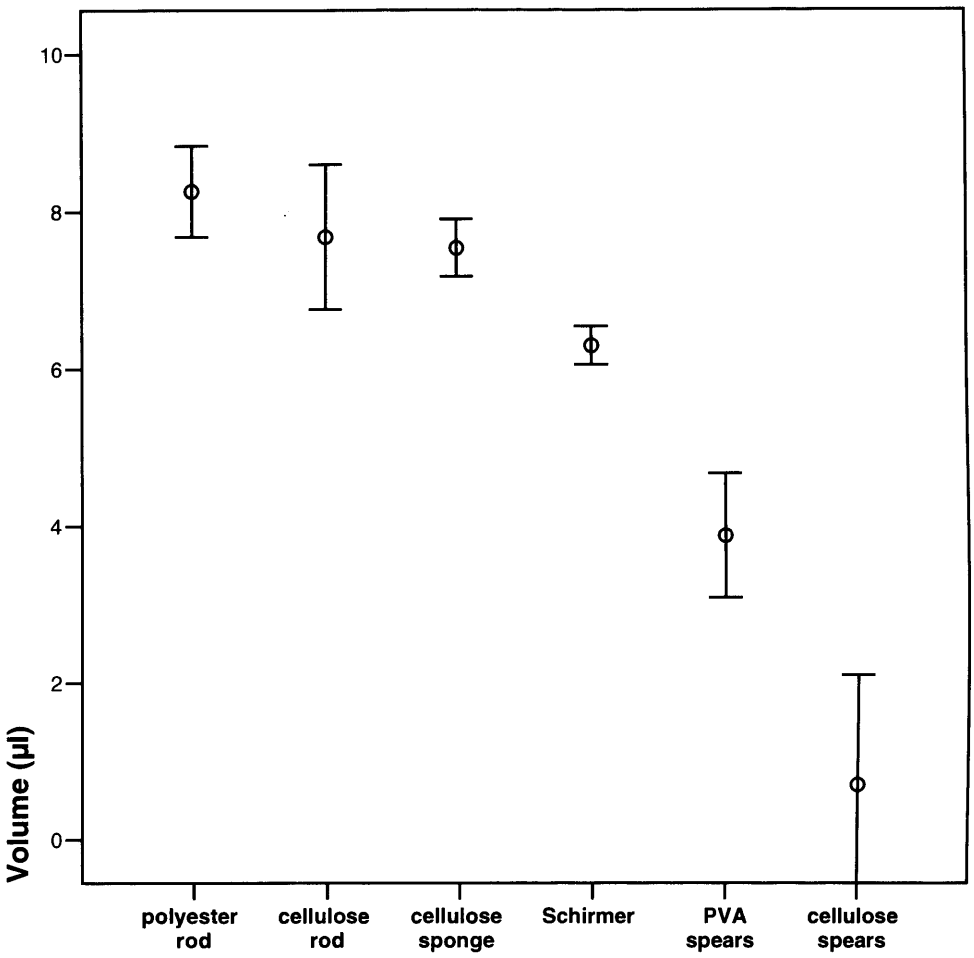


Figure 2.6 Results of tear volumes extracted from different test materials (µl, means±sd)

2.2.3 Conclusion

The tested collection materials can be differentiated in two ways: rigid or non-rigid, and natural or synthetic. Based on the statistical results and observations of extracted volume sizes, it was concluded that the best extraction was achieved with the polyester rods. These rods were synthetic and similar in rigidity to the cellulose rods. The results for cellulose rod and cellulose sponge were overall very similar (p=0.9).

These were both natural materials. For further testing the viscose towels were chosen as the best soft material, as a comparison for the polyester rods.

Based on the confidence areas (95% confident that the interval mean ± 1.96x standard error encloses the true mean), overall an extraction rate of between 79% and 86% can be expected for the polyester rod. Cellulose sponges will allow between 73% and 78% extraction.

2.3 EXPERIMENT 2: The effect of absorbed solutions

2.3.1 Introduction

In addition to the Opti-Tears™ solution, two different test solutions were used to mimic the tear film. One solution (Hylo-Prompt™) contained eye drops and had a similar viscosity. Similar to the Opti-Tears™, it is often used when the ocular surface is compromised with diseases such as dry eye. The third solution (Sensitive Eyes™ Plus) was saline and was chosen because it provides water-like properties.

All of the solutions had important compositional differences, such as a different osmolarity. The eye drops have a lower osmolarity than the tear film to counteract any increase in osmolarity caused by aqueous evaporation in dry eyes (Aragona et al., 2002). All three solutions were pH-balanced. The main difference was the lubricative agent used in each of them.

2.3.2 Methods and materials

The following two solutions were compared to the Opti-Tears™:

- Hylo-Prompt™ Eye Drops

HYLO-Prompt (Ursapharm, Germany) is a preservative-free eye drop with a viscosity of 4-10 mPa.sec. It is used to provide moisture the ocular surface. According to the supplied information, 1ml solution contains 1mg sodium hyaluronate. This accounts for the viscosity of the solution. Sodium hyaluronate is also used for arthritis treatment as a supplement for endogenous hyaluronan. It is injected into the joints to act as a lubricant (Brandt, Smith and Simon, 2000). Sodium hyaluronate on the ocular surface has been shown to prolong the residence time of the eye drop (Snibson et al., 1990). Compared to other lubricants, it has a better protective effect against dryness and epithelial cell damage (Debbasch et al., 2002).

- Sensitive Eyes™ Plus Saline

Saline has almost the same surface tension and viscosity as water (Zhao and Wollmer, 1998). As indicated by the manufacturer (Bausch and Lomb, UK), the saline solution is used for contact lens rinsing before placement on the eye. It is a balanced salt solution that can also be used for diluting enzymatic tablets and in disinfection processes. Saline has been compared with visco-elastic solutions during a visual task. It does not have the same lubricative effect for decreasing discomfort and modifying the blink rate (Acosta, Gallar and Belmonte, 1999).

The two additional preparations were centrifuged according to Table 2.1. The results were compared to the results from the previous experiment with the Opti-Tears™ solution. Each material was tested five times with each solution.

Table 2.1 Centrifugation parameters for experiment 2

Examined factor	Spin Duration (minutes)	Spin Force (x g)	Temperature (°C)	Volume (µl)	Materials
Three tear mimicking solutions	10	15300	4	10	polyester rod/ cellulose sponge

2.3.3 Results

Results were checked for normality prior to statistical analysis by the appropriate tests. It was assumed that the extracted volume would differ according to the solution and to the centrifugation effect on each material. The extracted volumes of each solution, at a given duration, and at a given force were compared for both materials (ANOVA, univariate).

Overall there was a difference found between the effect of the solutions $p<0.001$. When the combination with materials was examined the test showed a high significance ($p<0.001$). The results presented in Figure 2.7 show that extracted volumes from the polyester rod had a higher volume when saline was used. This difference was examined for the polyester rod and confirmed statistically ($p=0.004$, Bonferroni adjusted significance level of $p=0.008$). Observation of all extraction volumes with the cellulose sponge found no significant difference.

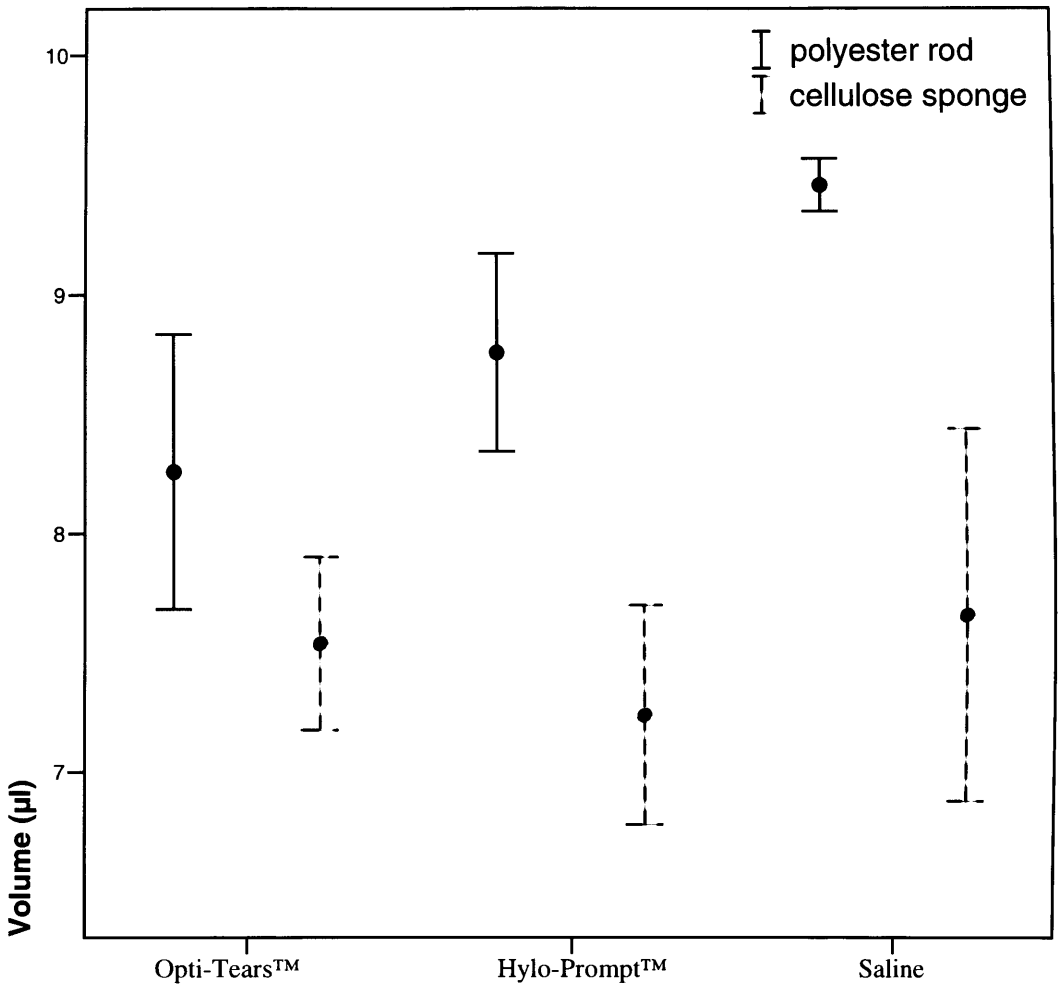


Figure 2.7 Volume extraction of different solutions (n=5 for each of the presented results)

2.3.4 Conclusion

This experiment showed that extraction volume from the polyester rod differes depending on the quality of the solution. Saline as the least viscose solution was more readily extractable.

2.4 EXPERIMENT 3: Centrifugation parameters

2.4.1 Introduction

Van Agtmaal et al. (van Agtmaal et al., 1987) reported on sample recovery in sponges and found centrifugation to be a reliable extraction method. The centrifuge (Boeco U-32 R, Germany) can vary the spin speed, and duration, and can control the temperature of the sample.

2.4.2 Methods and materials

For this study the spin duration was varied from 3 minutes to 30 minutes to assess the effect on the extracted volume. The spin speed can be expressed in two different ways: rotation per minute (RPM) and relative centrifugal force (RCF). RCF has units of "times gravity" (x g) and takes both RPM and the tip radius of the rotor into account. A conversion table can be found online at http://www.piercenet.com. In this chapter, the range was limited by the highest speed of 14000 RPM or a RCF of 15300 x g. The effect of two weaker forces of 700 x g and 5700 x g were also assessed.

A cooler temperature was preferred to protect the sample from heat produced by the rotation. To assess the effect of temperature in the centrifuge on the extracted amount, sample volume extracted was measured at room temperature (25°C) and again in a controlled cool temperature (4°C).

Using the Boeco centrifuge one parameter was changed in each trial, with the others kept at a standard value. Table 2.2 shows the factors that were altered (with a grey background) while the other factors in the same row remained unchanged.

Centrifuge Factors	Spin Duration (minutes)	Spin Force (x g)	Temperature (°C)	Volume (µl)
spin duration	3, 10, 20, 30	15300	4	10
spin force	10	700, 5700, 15300	4	10
temperature	10	15300	4, 25	10

Table 2.2 Different centrifuge parameters (left column) were tested by changing centrifuge modifications (top row) or the factors themselves (grey background) as in a one-factor-at-a-time-experiment (non-factorial).

2.4.3 Results

Results were checked for normality prior to statistical analysis by the appropriate tests. Results are presented for different centrifuge durations, force and temperature with each material. Statistical analysis included appropriate normality testing of the results.

2.4.3.1 Centrifuge duration

Results from the extraction study with different durations are shown in Table 2.3. The analysis (ANOVA, univariate) found no difference between durations (p=0.4) or between different materials (p=0.7).

Table 2.3 Extraction volume (µl; mean±sd) for each test material at different centrifugation durations (n=5 for each presented result)

Materials	3min	10min	20min	30min
polyester rod	8.3±0.68	8.2±1.24	8±1	7.98±0.66
cellulose sponge	7.6±0.3	7.44±0.39	7.61±0.3	7.76±0.49

2.4.3.2 Centrifuge force

As before with the centrifuge durations, the results for the extracted volume of each centrifuge force were observed with each material (Table 2.4). The ANOVA testing (univariate) showed no significant differences between the centrifuge forces ($p=0.5$). A difference was found between the materials ($p<0.001$). The observation of the results for the cellulose sponge showed an increase of extracted volume with higher centrifuge force.

Table 2.4 Extraction volume (µl; mean±sd) for each test material at different spin forces (n=5 for each presented result)

Materials	700x g	5700x g	15300x g
polyester rod	8.16±0.38	8.44±0.42	8.17±1.1
cellulose sponge	7.12±0.41	7.34±0.09	7.58±0.37

2.4.3.3 Temperature of centrifugation

The differences between the room and cooled temperature were tested with t-test (unpaired). The lower temperature had no significant effect on the extracted volume of the rods ($p=0.2$) and sponges ($p=0.8$). The results are shown in Table 2.5.

Table 2.5 Extraction volume (µl; mean±sd) for each test material, at 4 and 25°C (n=5 for each presented result)

Polyester rod		Cellulose sponge	
4°C	25°C	4°C	25°C
8.26±0.29	8.02± 0.26	7.54±0.18	7.58±0.22

2.4.4 Conclusion

Although the duration of centrifugation showed no difference with either collection material, all trials for the remaining factors were run for 10 minutes. This was because the analysis of the results was not carried out until after all experiments were finished. In the experimental design it was felt safer to use a moderately long duration. However, in future trials the spinning duration will be set at 3 minutes to save time. For the optimal extraction the rotation force at 15300x g was chosen as it allows higher extracted volumes. It was observed that by using a high centrifuge force heat is produced that can alter the samples. Centrifugation at 4°C has no effect on the amount of sample extracted from both materials, therefore the low temperature of 4°C was preferred.

2.5 EXPERIMENT 4: Effect of storage

2.5.1 Introduction

This experiment was conducted to assess the effect of a delay in sample extraction. This storage procedure was selected to evaluate a potential situation where a sample cannot be processed immediately. This may happen when there is a need for transportation of the sample.

2.5.2 Methods and materials

Samples were frozen at –20°C for 7 days. After this period, the frozen samples were kept for 30 minutes inside a fridge at 4°C to allow melting before centrifugation. All

centrifugation trials were run at 15300x g for 10 minutes at 4°C. The results for fresh samples were already obtained in Experiment 1.

2.5.3 Results

Results were checked for normality prior to statistical analysis by the appropriate tests. All results are presented in Table 2.6. The effect of storage was tested by t-test (unpaired) for each material. For the polyester rod a significant difference was found between fresh and stored samples (p=0.01). The means of the extracted volumes showed a slight increase. The storage of samples in the cellulose sponge did not affect the extracted volume (p=0.3).

Table 2.6 Extraction volume (µl; mean±sd) for each test material and Opti-Tears™, before and after storage (n=5 for each presented result)

Polyester rod		Cellulose sponge	
fresh	**stored**	**fresh**	**stored**
8.26±0.29	9±0.4	7.54±0.18	7.8±0.45

The increase of volume in polyester rod samples needed further investigation. To test if humidity can leak into the Eppendorf tube while it is under storage, the experiment was repeated with both collection materials. This time they were frozen and stored for 7 days without containing any solution. Both before and after centrifugation, no condensation was observed.

2.5.4 Conclusion

Storage at -20C° for 1 week may enhance the volume size for extraction in polyester rods.

2.6 EXPERIMENT 5: Effect of UV radiation

2.6.1 Introduction

For sterilisation, the test materials were exposed to UV radiation.

2.6.2 Methods and materials

The manufacturer guidelines recommend a period of at least 5 minutes to achieve a sterile effect. The test materials were placed into the radiation device (Ultra-Violet Products Ltd., UK) together with forceps and transparent plastic tubes for storage. The extraction rate from the sterile materials was compared to non-treated materials.

All centrifugation trials for the sterile samples were run at 15300x g for 10 minutes at 4°C. The results for non-sterile samples were already obtained in Experiment 1.

2.6.3 Results

Both collection materials were exposed for 5 minutes to UV radiation for sterilisation. To show that the procedure didn't alter the structure and absorption properties of the materials, the extraction was compared with materials not exposed to UV radiation. Results were checked for normality prior to statistical analysis by the appropriate tests. Results are presented in Table 2.7. The t-test (unpaired) was performed, testing

for the effect of radiation. This was shown to be not significant for both (polyester rod p=0.07; cellulose rod p=0.6).

Table 2.7 Extraction volume (µl; mean±sd) for each test material and Opti-Tears™, before and after sterilisation (n=5 for each presented result)

Polyester rod		Cellulose sponge	
non- sterile	sterile	non- sterile	sterile
8.26±0.29	8.72± 0.4	7.54±0.18	7.52±0.27

2.6.4 Conclusion

Treating the collection materials with UV radiation was shown not to change their extraction properties and is a suitable technique for sterilising the surface of the collection material prior to touching the ocular surface.

2.7 EXPERIMENT 6: Suitability for protein analysis

2.7.1 Introduction

The final experiment was designed to confirm that the ideal material is functional for biochemical tests. These tests are described only briefly. The assays used for tear analysis will be presented and discussed in more detail in Chapter 3.

Since samples extracted from the collection technique will be used for assessment of protein concentration and composition, it was essential that the technique chosen did not interfere with the assay. This was assessed by examining the effect on the total tear protein assay (Bradford test) and protein profile analysis (gel electrophoresis). Instead of the previous solutions, PBS (Phosphate buffered saline tablets, Sigma-Aldrich Co, UK) was used. PBS is a buffer that is used when tear samples need to be diluted. A volume of 10μl was subjected to the test material. Three materials were examined: polyester rod, cellulose sponge and the cellulose rod. The cellulose rod came from the same suppliers as the polyester rod, was used to confirm if effects were due to material substance. The test materials were treated in the same way as described in the previous two sections. PBS was extracted with the established ideal centrifugation method and used as a zero standard to confirm that the extracted volume did not mimic a protein.

2.7.2 Methods and materials

When the Bradford test was performed the extracted PBS was compared to PBS before extraction and bovine serum albumin (BSA). BSA was the provided protein standard that was used for the assay kit. It is used in gradual dilutions which react at different intensities with the provided reagent. The reagent changes its colour from brown into blue at the presence of any protein. To assess if the PBS mimics a protein after extraction, the colour of the reaction with the detergent is compared to the standard dilutions.

The extracted PBS was also subjected to gel electrophoresis and silver staining. Silver stain reacts with a present protein and band becomes visible with a stronger intensity than the background. BSA was used as a control protein.

2.7.3 Results

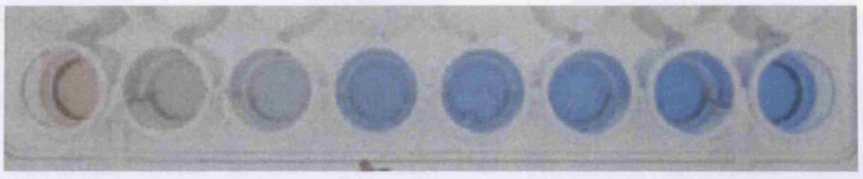
These are the results from both tests for contamination from the test materials: the Bradford test and gel electrophoresis.

2.7.3.1 Bradford test

PBS was extracted and subjected to the reagent. BSA was used as a positive control for protein staining. BSA dilutions from zero to 2μg/μl were plotted to show a change of the reagent into a blue colour by a true protein and compare the intensity with the sample result (Figure 2.8a). The first well contains PBS before use. Since no protein was present, the colour of the test reagent stayed brown. Samples from the test materials were subjected to the same reagent. Samples from polyester rod surprisingly reacted with the reagent (Figure 2.8b, second well) whereas extracted samples from the other materials did not stain blue.

Figure 2.8 Observations with the Bradford test

a) The reagent turns blue from brown (left well) with standard dilution of bovine serum albumin, The range of concentration is 0 to 2 µg/µl



b) observed colour effect of extracted PBS from the polyester rod on the right compared to no sample in the left well:

2.7.3.2 Gel electrophoresis

Samples from polyester rod, cellulose rod and cellulose sponge were subjected to gel electrophoresis and stained with silver staining. No protein-like staining was detected.

2.7.4 Conclusion

The polyester rod adds a contaminant to the sample that produces a positive protein result. Gel electrophoresis confirmed that no protein was present. Cellulose rod and sponge did not show any protein mimicking effect with both assays.

2.8 DISCUSSION

This study found that the polyester rod had the best extraction properties among the absorbent materials tested. However, the polyester rod proved to contaminate the sample, making it unsuitable for protein assays. Further, when used for collecting tears, it is possible that the contaminant would leak onto the ocular surface to cause a potential chemical insult to the eye. Of the remaining absorbent materials, the cellulose sponge showed the same efficiency as the polyester rod with respect to centrifugation temperature, sterilisation and storage. However, in comparison to the sponge, the ease of handling the rods and their shape made it more suitable for avoiding contact with the ocular surface during tear collection. Therefore, it was determined that the ideal material is the cellulose rod, in the optimal shape and size.

This study also defined the best centrifugation modalities for spin duration and force. Under these conditions all solutions were extracted similarly. The first two solutions were chosen because of their similar viscosity characteristics to the full tear film. Saline represented only the aqueous proportion of the tear fluid. Tear film is a very complex fluid and it is speculative to say which solution's absorption and extraction behaviour resembles it most. The results predict that after extraction the sample will be at least 70% of the originally collected volume.

No other study has previously considered the effect of centrifugation on extraction of collected fluids in different absorbent materials. There are no studies comparing extraction properties of materials or effects of storage and sterilisation. Only a few studies investigated protein recovery from absorbent cellulose materials (van Agtmaal et al., 1987; Tuft and Dart, 1989). The polyester rods used in this study have been

investigated before. Jones et al. (Jones et al., 1997) have compared polyester rods to glass capillary tubes. They collected tear samples and found the polyester rods 3.9 fold faster than the standard collection method. They used the rods also for protein analysis but did not test for contamination.

There are many other requirements for the ideal tear collection method (Jones et al., 1998). The most important application requirement when collecting tears, particularly from infants, is safety. This is ensured with better control of the material. Some materials proved easy to handle with forceps, such as both rods. Craig and Blades (Craig and Blades, 1999) have already described the mechanical irritation to the ocular surface by Schirmer paper strips so that round shaped materials and soft spongy materials should be preferred collection materials.

In Chapter 4 the absorbance efficiency of the cellulose rods on tear fluid was determined and compared to capillary tubes. The effect on protein analysis and reproducibility of results and safety of the method are further requirements to establish the cellulose rod as an alternative method to the capillary tube.

3 Tear analysis methods

In this thesis, infant tears were examined in two different ways. They were analysed by their ferning pattern and by their protein profile biochemically.

3.1 SAMPLE TREATMENT BEFORE ANALYSIS

Centrifugation is a well-controlled method for the purpose of sample recovery. It is used to filter out cells and release the proteins in a fluid. For tear fluid, it is most commonly used to remove cells (Kuizenga et al., 1990; Berry, Harris and Corfield, 2003). In the following studies, small non-stimulated samples were available. In these studies, centrifugation was only used to extract fluid from the absorbent material. The determination of the optimal spinning duration and force for extracting the most amount of fluid was described in Chapter 2.

Within the short amount of time before analysis, the samples were kept on ice (temperature between 0 and 5°C). This reduced the risk of alterations in the protein content due to temperature changes (Sitaramamma et al., 1998a; Schmut et al., 2002).

3.2 TEAR FERN TEST

In this study the tear fern test was used to investigate if infant tears show a pattern that resembles the tear fern from subjects with dry eyes. As described in the literature review, the ferning pattern of a dried tear drop can be viewed under a microscope and graded according to Rolando (Rolando, 1984). This grading differentiates between

tear fern patterns from subjects with normal tear film most of them falling into grade I and II and subjects with dry eye syndrome mostly with grade III and IV.

The process of tear ferning was easy and took less than five minutes. To obtain a small amount of sample the tip of the micropipette was dipped into the sample. A very small drop with a volume <0.1µl was observed at the tip. This drop was then pushed out when the tip touched the microscope glass slide. All microscope glass slides were cleaned with ethanol prior to use. The drop was spread out with the edge of a cover glass and became opaque as soon as it dried on the slide. To avoid any effect from forced drying, the plate was kept isolated from wind or draft. Other variations, such as using two slides with the drop in between or dilution of the sample with PBS did not show any improvement of the method. The drop was viewed under the microscope. It was viewed at 20x, 40x and if resolution allowed at 60x magnification. The image was graded and saved on computer.

To test if the sample handling affects the ferning, saline was used as a sample to insert and extract from the cellulose rod and the capillary tube. The extracted saline was dried and viewed as described above. No additional material was seen in the dried drop area.

3.3 BIOCHEMICAL ANALYSIS

One of the aims of this thesis was to analyse infant tears using biochemical techniques (e.g. electrophoresis and chromatography) that have been used previousely for analysing tear protein profiles (Boukes et al., 1987; Fullard and Snyder, 1990; Schmut et al., 2002; Grus et al., 2005). Chromatography typically requires a minimum of 5µl tears (Fullard, 1988) and as this volume was unlikely, especially with neonates, the technique of gel electrophoresis was used to screen the tear samples. Additionally, the total tear protein concentration was determined. Both methods are easy to perform, cost-effective and appropriate for a small sample size (Glasson et al., 1998; Li et al., 2005). Gel electrophoresis is a semi-quantitative technique and permitted verification of found proteins by Western blotting.

This chapter discusses these biochemical methods and their optimisation, starting with the sample treatment after collection, before moving to the total tear protein measurement, focusing on the Bradford test as the assay of choice. After determining the total tear protein, the sample was subjected to gel electrophoresis. The method for gel electrophoresis is described, including

- sample preparation,
- equipment,
- preparing the gel,
- running the assay,
- protein standards,
- sample loading
- reduction of the background intensity, and finally

- densitometry is described.

This chapter finishes with the description of Western blotting, such as

- materials and solutions used,
- the transfer of proteins from gel electrophoresis, and
- the process of Western blotting.

3.3.1 Total tear protein concentration analysis

Total protein concentration describes the combined concentration of all proteins. The measurement technique is not specifically sensitive to one protein or group of proteins. Therefore colorimetric techniques are used that measure the colour change and density of a dye after it binds to proteins. Several techniques exist for this purpose such as the BCA (bicinchoninic acid) protein assay (Smith et al., 1985), Lowry protein assay (Lowry et al., 1951) and the Bradford method (Bradford, 1976). It has been shown that the results from different techniques cannot be compared with each other (Ng et al., 2000).

All the studies in this thesis measured the total protein concentration by the Bradford method. This form of assay is easy, fast and often recommended research technique (Kijlstra, Jeurissen and Koning, 1983; Dartt, 1989; Stolwijk et al., 1994; Sitaramamma et al., 1998a) for analysis of tear protein secretion. It utilises Coomassie brilliant blue G250 (or Coomassie blue), which converts its light absorption from 465 to 595 nm upon binding to proteins in solution.

Total tear protein was measured to establish the effect of the different collection techniques and the effect of storage on the tear sample. It was also used to examine the protein concentration for different experimental groups, such as adults and neonates.

3.3.1.1 The Bradford test

Bradford developed this rapid and sensitive protein assay based on the observation that the dye, Coomassie blue, is converted from a red/brown colour to blue upon binding to proteins (Bradford, 1976). The main advantage is the low interference with non-proteins (Walker, 2002).

Samples were assayed for total tear protein concentration using the Coomassie Plus™- The Better Bradford Assay kit (Pierce Biotechnology Inc, USA). This assay consists of a kit that provides a ready-to-use working reagent and a protein standard. It works based on the Bradford test. The reagent contains the dye, Coomassie blue, already dissolved in ethanol and phosphoric acid. This means it can be used faster and does not involve many preparation steps. The sample needs to be added to the reagent and the reaction between the reagent and the proteins in the sample changes the colour of the dye. After incubation the mix solution can be analysed by measuring the light absorption intensity and comparing it to a standard. At the time of its purchase this assay kit had the shortest incubation time compared to other commercially available Bradford assays (Mok et al., 2004).

The manufacturer's instructions (Pierce Biotechnology Inc, USA) offered different protocols for the assay. In this thesis the procedure followed the standard microplate

protocol for a working range of 0.1-1.5μg/μl. This form of assay was used because of the limited sample volume. In addition it is known to be more sensitive to proteins (Walker, 2002).

1. Prior to use the reagent and the samples were allowed to acclimatise at room temperature for 30 minutes. The instrument for measuring optical densities was a plate reader (Multiskan Ascent 96 Plate Reader, Thermo Electron Corporation, UK) which was allowed to warm up for at least 20 minutes. For better transmission of light, its light source needs to be warm before use.

2. The tear sample was diluted to contain a protein concentration appropriate for the working range of the kit. The accurate dilution of tear samples was determined by a series of dilutions (increasing the dilution factor) of pooled tear samples, so that the resulting absorbance fell within the linear range of the assay. Tear samples were diluted with PBS (Phosphate buffered saline tablets, Sigma-Aldrich Co, UK). The dilution factor for tear samples was 1:10 and a mix volume of 10μl was pipetted into one of the 96 wells of the microplate (Fisher Scientific, UK). This means that each used well contained 1μl tears.

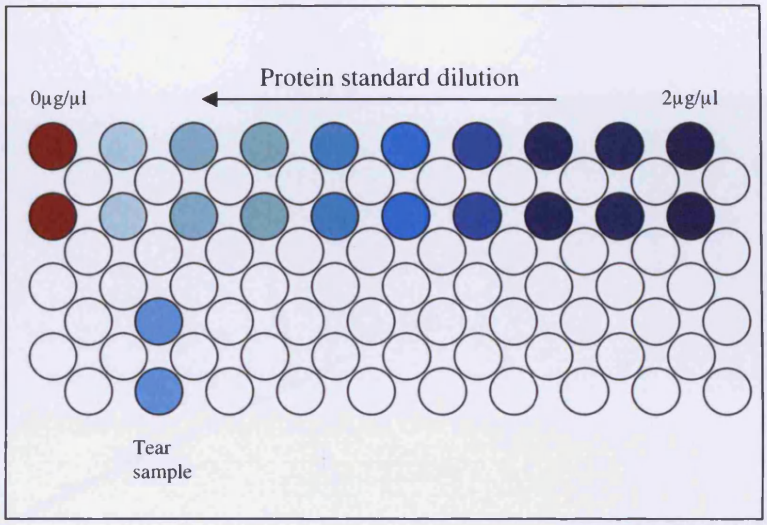
3. Sample concentrations were determined from a bovine serum albumin (BSA) (Sigma-Aldrich Co, UK) standard curve created by diluting a stock solution of 2μg/μl BSA with PBS. A minimum of 10 dilutions, resulting in a concentration range between 0 and 2μg/μl were used. 10μl of BSA standard mix were pipetted into the wells.

4. Although this was not mentioned by the protocol, if sufficient amounts of volume were available, each sample and standard was used twice and results were averaged.

5. Three hundred µl of a supplied ready to use reagent was added into each well. The tear sample or BSA standard solution was mixed with the reagent. This part of the standard protocol was slightly altered after the first trial by changing the order of mixing. The reagent was first pipetted into the wells, and then the samples and standard were added to it. The pipette tip was held into the reagent and the sample/reagent mix was sucked in and out of the pipette to ensure that it has entirely been expressed into the reagent.

6. To mix the reagent with the standard or sample, the plate was slowly shaken for 30 seconds in the ELISA reader (Multiskan Ascent, Thermo Electron Corporation, UK) and left incubating at room temperature for ten minutes. During this time the wells were covered to avoid evaporation. In the presence of the proteins the colour of the reagent changes from brown to blue (Figure 3.1). The incubation was necessary for a complete binding process of dye and the proteins.

Figure 3.1 A schematic view of the Bradford assay. The wells were filled with reagent, protein standards and tear samples. This figure shows how the colours may change after reaction with the reagent. The optical density of the absorption at 595nm was measured for the tear sample and compared to the protein standard dilution.



7. The supplier of the assay kit recommended measuring absorbance at or near 595nm. The intensity is measured simultaneously at the absorbance of 590nm, as this was the nearest possible absorbency option of the plate reader.

8. The well that was filled with PBS (0µg/µl standard BSA) was used as a Blank. The average measurement for the Blank was subtracted from all other individual standard and sample replicates.

9. Average Blank-corrected 590nm measurements for each BSA standard were plotted versus the concentration in µg/µl (Figure 3.2). This plot was done in Excel (Microsoft, USA). The best-fit curve was linear, and Excel provided an

equation with y= ax + b to convert measurements (y) into concentrations of µg/µl (x). These were than multiplied with the dilution factor for the final result.

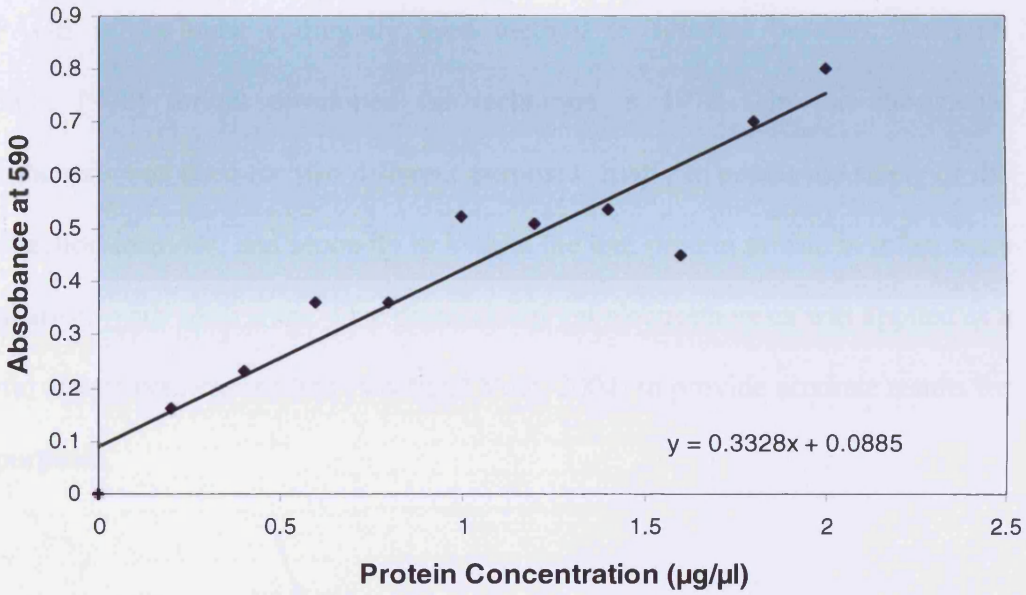


Figure 3.2 Example of a standard curve and the equation for converting measured absorbencies into protein concentrations

10. If a sufficient sample volume was not available, the standard protocol was modified. Only 0.5µl sample volume was diluted in a 1:20 dilution factor with PBS. If the measurement was within the working area concentration, this result was used as accurate.

3.3.2 Assessing the protein profile- Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The tear protein profile and the concentration of individual proteins were determined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). SDS-PAGE is the most commonly used method to separate proteins. Laemmli (Laemmli, 1970) further developed the technique in 1970. In this thesis, gel electrophoresis was used for two different purposes: firstly to assess the safety of the tear collection methods, and secondly to look at the tear protein profile in infant tears in comparison with adult tears. One dimensional gel electrophoresis was applied as a powerful and convenient method (Voet and Voet, 2004) to provide accurate results for these purposes.

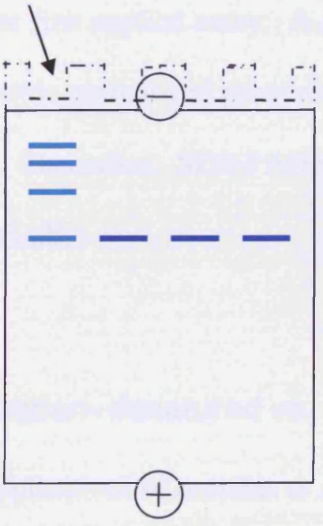


Figure 3.3 A schematic view of protein separation by SDS-PAGE. The gel is connected to an electrical field. The samples and the marker have already been subjected to the wells of the stacking gel (arrow) and are running in separate lanes. On the left is the marker that can be used prestained (e.g. light blue) to make individual proteins with known molecular size visible. Samples contain a stain (dark blue) that makes them visible before they reach the bottom of the gel.

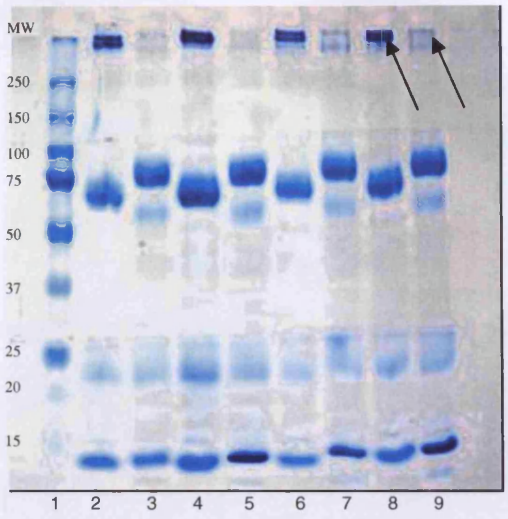
SDS-PAGE uses the fact that proteins are charged molecules and respond to an electric field. They can thus be made to move on a medium, such as a paper or gel. Through the electrical force the protein moves from the cathode electrode to the anode electrode (Figure 3.3). The mobility of the protein is determined by the pores in the gel. The polyacrylamide concentration in the gel determines the pore size so that the gel serves as a sieve to separate proteins by their mass. At a given pH, the larger molecules move less quickly than the small molecules move through the gel. The separated proteins are located according to their molecular weight. The SDS-PAGE provides protein purification with high resolution, which can be enhanced further by combination with Isoelectric Focussing. This is an electrophoretical method that separates proteins on the basis of acidic and basic residues. The extension of isoelectric focussing by SDS-PAGE is called a Two-Dimensional Electrophoresis. Isoelectric focussing is the first applied assay. A pH gradient is set up in the gel, the gel is subjected to the electric current and proteins move according to their charges to their isoelectric point. Thereafter, SDS-PAGE is applied in the perpendicular direction to separate the bands.

3.3.2.1 Sample preparation- denatured vs. non-denatured

There are two different applications of proteins to a gel, denatured and non-denatured. Some investigators (Grus et al., 2002; Lopez-Cisternas et al., 2006) denature the tear proteins to obtain a less complex shape. The protein loses its secondary and higher order structure, stabilised by different bonds, and is changed in its migration on a gel (Schmut et al., 2002). When proteins are denatured prior to applications; the sample is dissolved in a buffer with SDS and detergents such as beta mercaptoethanol or DTT (Hames and Rickwood, 1990; Streyer, 1999). The solution can be incubated at room

temperature, or for a faster process is heated at 100°C. The detergent dissolves disulfide bonds such that the protein is evenly coated with the SDS. The resulting protein has a negative charge and migrates in the gel relative only to its size and not its charge or shape.

Kuizenga et al. (Kuizenga, van Haeringen and Kijlstra, 1991) examined the standard protocol for denaturing and described how this sample treatment affects the secretory IgA. After denaturing the sample, the large IgA molecule (400kD) results in three fragments, the secretory component, the heavy and the light chain. The band for the secretory component was then co-migrating with the lactoferrin band. To achieve a clearer separation of IgA, lactoferrin, and serum albumin in this thesis, the sample stayed non-denatured. Sample treatment followed the protocol and concentrations described by Ng et al. (Ng et al., 2000). The tear sample was added to a sample buffer containing SDS and bromophenol blue in a ratio of 1:3. Bromophenol blue is a stain to make the movement of the sample on the gel visible. This sample preparation was completed five to ten minutes prior to use.



1	Marker
2; 4; 6; 8	non-denatured samples
3; 5; 7; 9	denatured sample

Figure 3.4 Tear samples were subjected with and without denaturing on an SDS-gel, to test the location of proteins, such as IgA (arrow) according to this sample treatment.

Figure 3.4 shows some denatured and non-denatured samples applied for different subjects. Serum albumin is only detectable in the silver stained gel with non-denatured samples. IgA disappears from the top after denaturing.

3.3.2.2 The equipment

A vertical mini-system (GE Healthcare UK Ltd, Amersham Biosciences, UK) (Figure 3.5) was used. To prepare the gel, a glass plate and a plastic plate with 0.75mm thick spacers were assembled in a clamp on a casting stand. The space between the plates was first filled with 100% ethanol to test that the assembly does not leak at the bottom.

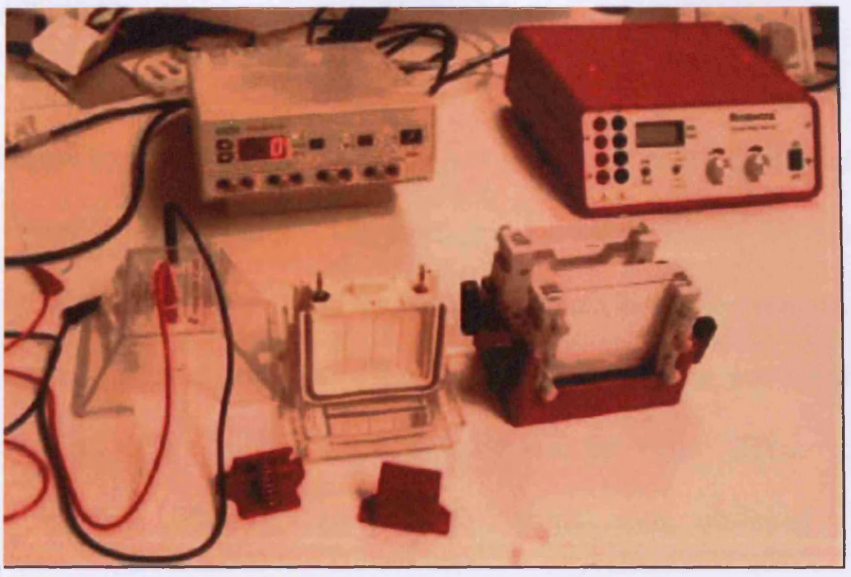


Figure 3.5 Gel casting, mini-system equipment for gel electrophoresis (GE Healthcare UK Ltd, Amersham Biosciences, UK)

3.3.2.3 Gel preparation

This is the standard procedure for gel preparation. Gels were mixed according to the concentrations in Table 3.1 (Sambrock, Fritsch and Maniatis, 1989). The gel solution was mixed and filled with a 5ml plastic pipette. At the top a 1.5cm gap was left for the stacking gel. The stacking gel was added after a 30 minutes delay to allow for the separating gel to become firm. A 0.75mm comb with 15 wells was immediately inserted into the stacking gel to produce wells. The stacking gel served to concentrate the protein mix. Depending on the number of wells, several samples of each 15-20µl could be filled for each gel.

After polymerisation, gels were transferred to the vertical slab gel unit and fixed with clamps. Running buffer (10xTris/Glycine/SDS buffer from Bio-Rad Laboratories

Ltd., UK, and diluted 1:10 with distilled water) was added to the top of the gel until each well was filled.

To achieve a better separation, generally gels were used with a gradual change in their polyacrylamide concentration. Therefore gels for measuring the serum albumin were produced in the lab from 8-12%, i..e., three resolving gels were produced: a 12% gel for the lowest part, then 10% and 8%. Each gel concentration was inserted and left to dry before the next resolving gel was filled into the cast. Within one gel, at the borders between different resolving gels, bands were observed after staining. Unfortunately this sometimes meant that bands would appear at the border. As a consequence the gels used for major tear proteins were prepared conventionally, 10% separating gels.

At first, when looking at the serum albumin no stacking gel was necessary, but once the profile of all proteins were analysed the stacking gel was added.

Table 3.1 Solutions for preparing for SDS-PAGE (Sambrock et al., 1989)

Component volumes (ml) for 10ml resolving and 3 ml stacking gel solution components	Resolving gel 8%	Resolving gel 10%	Resolving gel 12%	Stacking gel 5%
distilled water	4.6	4	3.3	1.4
30% acrylamide	2.7	3.3	4	0.33
1.5M Tris/HCL pH 8.8	2.5	2.5	2.5	
1.0M Tris/HCL pH 6.8				0.25
10% SDS	0.1	0.1	0.1	0.02
10% APS	0.1	0.1	0.1	0.02
TEMED	0.006	0.004	0.004	0.002

3.3.2.4 Identification of proteins

There are generally three standard ways for the identification of the protein bands:

- by comparison of the migration of the marker with the individual proteins. This enables the assessment of a protein's molecular weights;
- by the comparison of bands to bands of known standards, subjected concurrently to serve as control proteins (Kijlstra et al., 1989; Kuizenga et al., 1991; Ng et al., 2000) and
- by comparison to known proteins (Fullard and Snyder, 1990; Sack et al., 1992; Ng et al., 2000).

Molecular weight markers

Markers contain several proteins, each representing a different molecular weight. Many different markers are commercially available. They can be different in the range of molecular weights and in the staining method. In this thesis, a prestained blue marker (Precision Plus Protein Standard from Bio-Rad Laboratories Ltd., UK), with a molecular weight ranging between 250 and 10 kilo Daltons (kDa) was used (Figure 3.6).

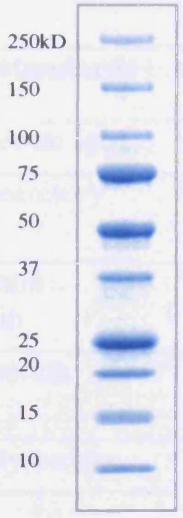


Figure 3.6 The molecular weight markers from Bio-Rad Laboratories Ltd., UK as depicted on their website (www.bio-Rad.com) was used to identify the proteins by their molecular weight on the gel and on the Western blotting. To the left is the molecular weights range in kiloDaltons (kD).

The prestaining allowed observation of the sample migration and its spread. A volume of 10µl was subjected to the gel and on a 10% gel the marker stretched between 250 and 15 kDa.

Protein standards

A marker consists of a mix of different standards, known only to the supplier. It is possible that proteins in the sample with the same molecular weights as in the marker migrate at a different weight due to different preparation and treatment (see section 3.3.2.1 Sample preparation- denatured vs. non-denatured). In addition, known protein standards may confirm the identification and allow quantification of the protein bands (Table 3.2).

Protein standards	**molecular weight**	**Used concentration**	**supplier**
human serum IgM	900kD	1 μg/μl	Autogen Bioclear, UK
human secretory IgA	395kD	1.8μg/μl	Sigma-Aldrich Co, UK
human milk lactoferrin	90kD	2.5μg/μl	Sigma-Aldrich Co, UK
Human serum albumin	66.5kD	0.5μg/μl	Sigma-Aldrich Co, UK
hen egg lysozyme	14.7kD	2.5μg/μl	Sigma-Aldrich Co, UK

Table 3.2 Standards used and their concentrations

Hen egg lysozyme has the same molecular weight as human lysozyme and has served as an alternative before (Bonavida et al., 1969; Hankiewicz and Swierczek, 1974). The only commercially available IgM standard (Autogen Bioclear, UK) was produced for ELISA assays and was not recommended as a useful reference for quantification purposes. It was only used to find its position on the SDS-PAGE and re-confirmation by Western blotting.

Usually each protein is compared against its own standard and each protein standard is subjected to a separate lane. However, by using many different standards, the number of wells left for samples would become limited. To solve this, it was decided to run multiple standards in one well. All the protein standards were first run in different wells to show if there was any impurity. This was because extra protein band(s) are seen in the gel when the standard is contaminated with impurities. There was no impurity found that would overlap with the bands of another standard. A volume of 0.5µl of each standard was mixed and subjected to one well.

In order to know which concentrations are suitable, each reference standard protein was run in serial dilutions. Then, the intensities of the protein bands were compared to the corresponding protein in pooled tear samples. Additionally the standard concentrations were chosen by consulting the literature (Fullard and Snyder, 1990; Sack et al., 1992; Ng et al., 2000). The measurement technique will be described in detail in section 3.3.2.9 Measurement by densitometry.

3.3.2.5 Sample loading

Sample loading followed the standard protocol. The samples were loaded with micropipette (range 0.5-10µl, Biohit, UK) and an especially fine tip (Seque/Pro Capillary Pipet Tips, Bio-Rad Laboratories Ltd., UK) (Figure 3.7). As was done for the Bradford test (see section 3.3.1.1 The Bradford test), to increase accuracy, each sample with sufficient volume was duplicated on the same gel and the intensity measurements were averaged.

Each lane contained 0.25µl and 0.5µl tear sample for the silver and blue stain, respectively. Power supply came from Biometra standard Power pack P25 (Anachem, Luton, UK) and was attached to the lid of the unit. The samples were run at a constant current of 30mA for approximately 90 minutes. Once the visible dye in the sample buffer approached the bottom, the gel was ready for washing and staining or Western blotting.

Figure 3.7 Pipette tips for gel loading in two different sizes (Bio-Rad Laboratories Ltd., UK). The smaller size was used for loading tear samples, while the larger tip was used for loading the marker

3.3.2.6 Used staining methods

To assess that no contamination from the absorbent material was present and for monitoring the serum albumin concentration in tears, a silver stain kit (SilverSNAP stain kit, Pierce Biotechnology Inc, USA) was used. For determining the major tear proteins, gels were stained with blue stain (Pierce Biotechnology Inc, USA). This

section presents an overview of standard staining options and a description of stains used.

Staining reveals a sequence of protein bands. Two types of stains were used for this thesis, a silver stain and a blue stain. They were chosen depending on the required sensitivity. The highest sensitivity can be achieved when silver staining is carried out. The smallest sample concentration detectable is approximately 0.001µg (Stryer, 1999). When silver stain was used for this thesis, it was observed that it did not stain regularly, so that the colour slightly altered between different proteins (Figure 3.8). This meant that silver stain should only be used for quantification with careful interpretation of the results. In this thesis it was only used to visualise the less abundant serum albumin.

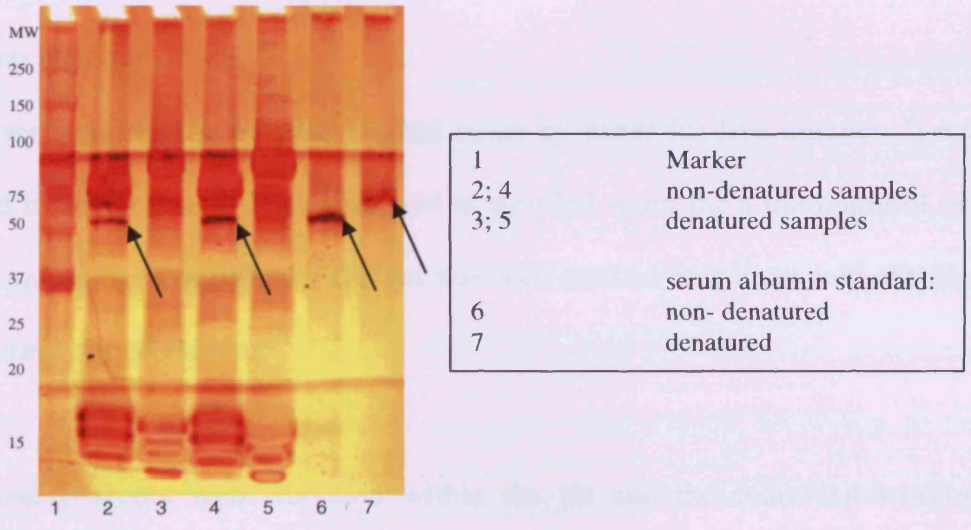


Figure 3.8 Denatured and non-denatured samples on an SDS-gel, to test the colour and sensitivity of the silver stain for the less abundant serum albumin (arrow).

Serum albumin standard from human serum with a concentration of 0.5µg/ µl shows that silver stain is better at detecting low concentrations.

The observation of blue stain showed an even colour for each individual protein. Although blue stain is less sensitive than silver stain (0,008µg), its stable colour makes it more suitable for protein quantification.

Alternatively, blue stains can be used for visualisation. The most commonly used blue colour stain is Coomassie blue, such as that used for the Bradford test. The blue stain used for the gel staining was a modified coomassie blue stain (according to Pierce Biotechnology Inc, USA) and was used for determining major tear proteins, such as lactoferrin and lysozyme.

3.3.2.7 Staining protocols

- Silver staining

Prior to silver staining, the gel was washed twice by water for five minutes. It was then fixated in 50% ethanol, 5% acetic acid in distilled water for a minimum of one hour or left in fixation over night. The gel was then washed four times with distilled water, each time for 30 minutes.

Four different reagents were supplied within the kit and the following working solutions were used as instructed:

1. silver solution
2. reducer solution by combining an aldehyde and base solution
3. stabiliser base solution

The gel was incubated in the silver solution for 30 minutes, washed for a few seconds, and then incubated in the reducer solution for five minutes. Finally the gel was incubated for at least five minutes in the stabiliser solution.

- Blue staining

When blue stain was used, the gel was first rinsed with distilled water for 15 minutes. It was then left in the stain reagent, and after one hour the reagent was rinsed with distilled water.

3.3.2.8 Optimisation of the staining protocol

When silver stain was used with the standard protocol, gels showed a tendency to have dark backgrounds. A strong background colour makes measuring weak bands more difficult by introducing noise and decreasing sensitivity. For eliminating a dark background it was recommended to increase the water rinse after the different steps. Therefore, a small preliminary study was carried out with increased washing times and steps.

- Methods and materials

Pooled samples from different subjects were run on several gels and, after the fixation step, all gels were cut in 4 pieces. Some gels were stained according to the instructions and the pieces were rinsed at increasing durations (Table 3.3 Variations (1) to (4)). This was done after incubation in the silver solution. Other gels were additionally introduced to longer rinsing times (Table 3.3 Variations (5)-(8)) after the incubation in the reducer solution.

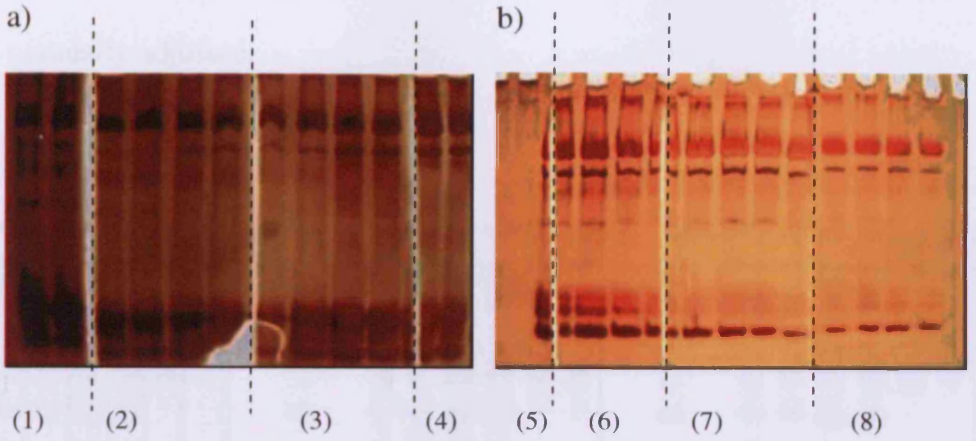
Variations of rinsing times	Water Rinse 1st step (seconds)	Water Rinse 2nd step (seconds)
(1)	5-10	0
(2)	20	0
(3)	40	0
(4)	60	0
(5)	20	10
(6)	20	20
(7)	40	40
(8)	40	60

Table 3.3 Instructions supplied by Pierce Biotechnology Inc, USA suggested rinsing times (row 1).

- Results

When the gels were rinsed only at water rinse 1 step (1-4), the decrease in background was very weak. Long rinsing times, such as in (7) and (8) were found to decrease the band intensities. Options (5) and (6) were therefore a good compromise between decreasing background colour and altering protein band staining intensity (Figure 3.9).

Figure 3.9 Gel background and protein bands with different rinsing times during the staining process



- Conclusion

A rinsing time of 10-20 seconds at both steps was chosen for an optimal staining protocol. For accurate and consistent staining, a timer was used for each SDS-PAGE.

3.3.2.9 Measurement by densitometry

All protein bands were measured by scanning densitometry (Epson Expression 1680 Pro, UK and Image Acquisition and Analysis Software, Ultra-Violet Products Ltd., UK). The gel was scanned and saved in a jpeg file format. It was then viewed with special software for gel image analysis that measured the band intensities in several steps. Figure 3.10 shows some of the main steps that are described here:

1. First all the required lanes were determined and marked
2. The lane with the marker was selected, bands were found automatically, and in a separate window a molecular weight was appointed to individual bands.

3. The background intensity of the bands was subtracted from each of the other lanes.
4. The bands were located automatically and the area around each band was manually adjusted.

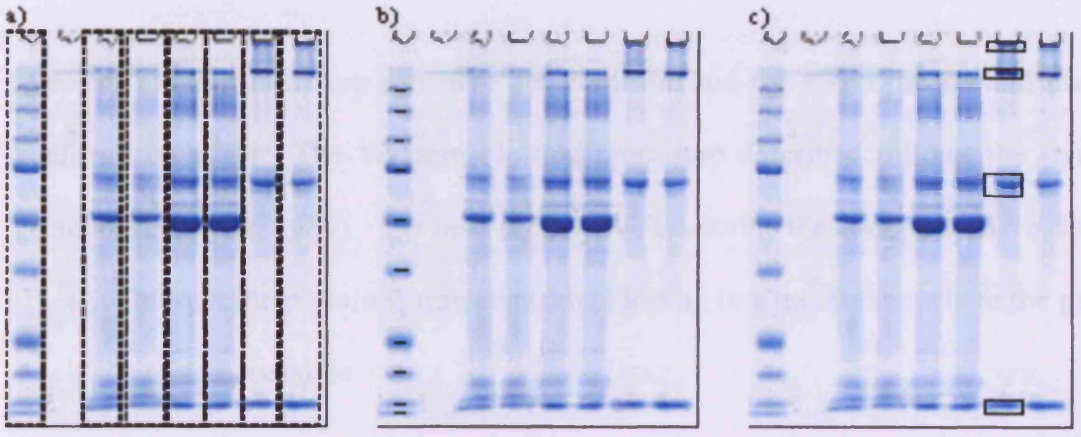


Figure 3.10 Some of the important steps in measurement of the band intensities by densitometry (a schematic depicturing of the software analysis):

a) by identifying lanes, b) appointing a molecular weight to each determined band of the marker, c) determining the area in which each lane is found (this is shown here for just one lane)

- Once all the bands were selected, their optical density (as the sum of the intensity within an area and peak height of each band) was measured and presented in a table, including the molecular weight.
- The table was converted into Excel and the ratio between individual protein band intensity and the standard was calculated. This result was used to calculate the protein concentration by plotting the ratio between the tear protein band intensity and its standard, against the ratios of the individual dilutions and the standard concentration used on the gel.

After scanning, the gels were kept sealed (for example in Tupperware) with distilled water.

3.3.3 Western blotting

The identity of the proteins that were closely located needed to be confirmed with the Western blotting technique. This was a necessary part of the proteomics of this thesis and it was generally expected that it was carried out on a minimum of three samples and on the protein standard.

Western blotting is a very sensitive detection method for a specific protein that has been run on a gel. The Western blotting procedure described follows the standard protocol (Burnette, 1981). The next sections will describe these steps in more detail:

1. The specific protein is transferred by blotting to a membrane where the protein is more accessible.
2. An antibody is added to build an antibody-antigen complex and this is detected by a second antibody.
3. The so called secondary antibody is radioactively labelled and produces a dark band image on an x-ray film.

For all these steps, several different buffers are necessary, such as a washing buffer for the washing steps. The content of these buffers and their amounts are described in the Appendix 3.

3.3.3.1 Protein transfer after SDS-PAGE

Available remaining tear samples were subjected to SDS-PAGE. Immediately after running the gel, proteins were transferred from the SDS-PAGE onto a nitrocellulose membrane (GE Healthcare UK Ltd, Amersham Biosciences, UK). The filter papers and the membrane were cut to the size of the gel and left in methanol for 30 minutes

to pre-wet. The methanol was exchanged with transfer buffer. A sandwich of two filter papers, one membrane, the gel and one filter paper was assembled on the blotting apparatus (Figure 3.11). The transfer was performed for a duration adequate for the protein size (20 min for Lactoferrin and serum albumin, 30 minutes for IgA, 45 minutes for IgM) at 0.05 V and 0.65mA/cm^2 on a Biometra standard Power pack P25 from Anachem, UK).

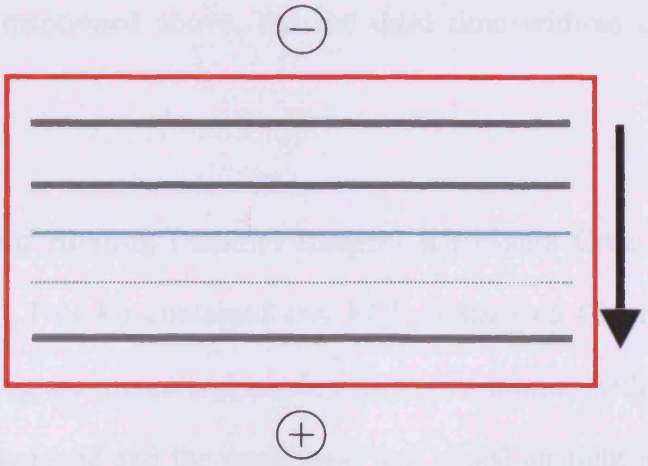


Figure 3.11 A schematic view of the order of filter papers (grey), SDS-PAGE (blue) and membrane (dashed) within the closed environment of the apparatus (red). The direction of current flow and movement of the proteins in the sandwich is shown by the arrow.

Once transfer was finished, the membrane was checked by Ponceau S staining buffer in a 1:10 dilution with water (Sigma-Aldrich Co, UK), a red stain, to determine if the range of proteins with the desired molecular weight were transferred. This stain was not permanent and was immediately washed off by distilled water. If the delicate gel did not tear it was stained with blue stain, as described above.

3.3.3.2 Incubation steps with first and second antibody

The membrane was washed three times with distilled water before immersing into 10ml blocking buffer for 30 minutes. The membrane was incubated with the primary antibody (dilution of 1:5000) overnight at 4°C. It was then washed three times with a washing buffer, each time for 10 minutes. In the next step, the membrane was incubated for one hour with the secondary antibody (dilution 1:1000). This antibody was also washed off three times, each time for 10 minutes. For washing, the same buffer was used as mentioned above, but the third time without containing milk powder (Appendix 3).

For detection, Western Blotting Luminol Reagent Kit (Santa Cruz Biotechnology, Germany) was used. This kit contained two ECL (Enhanced Chemiluminescence) reagents A and B that were mixed and incubated for one minute with the membrane. Excess reagent was removed and the membrane was sealed air tight in cling film. It was then placed into an X-ray cassette. The blot was visualised on X-ray film (Kodak Scientific Imaging film, Kodak, UK) for 30 minutes. The film was developed manually in standard solutions for developing and fixating (Sigma-Aldrich Co, UK)

If the same membrane needed to be used to verify that the particular protein did not interfere with another one, the used antibodies were stripped off in a stripping buffer for 30 minutes at 50°C. After stripping, the same procedure as described above was repeated for the different protein.

3.3.3.3 Results with Western blotting

Western blotting was used to detect IgA and to differentiate it from IgM and serum albumin. Primary monoclonal antibodies for these proteins were obtained from Sigma-Aldrich Co, UK. To detect lipocalin, a primary antibody against recombinant lipocalin 2 was obtained from R&D Systems, UK.

3.3.3.4 Serum albumin and IgA

Western blotting of human serum albumin and IgA was used to show that samples stayed non-denatured during sample process and that both proteins produced bands that did not overlap.

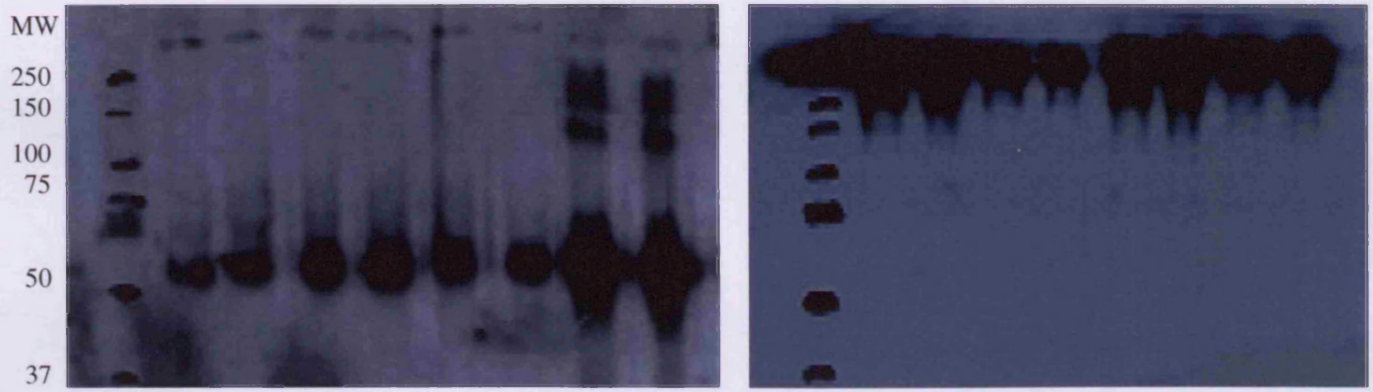


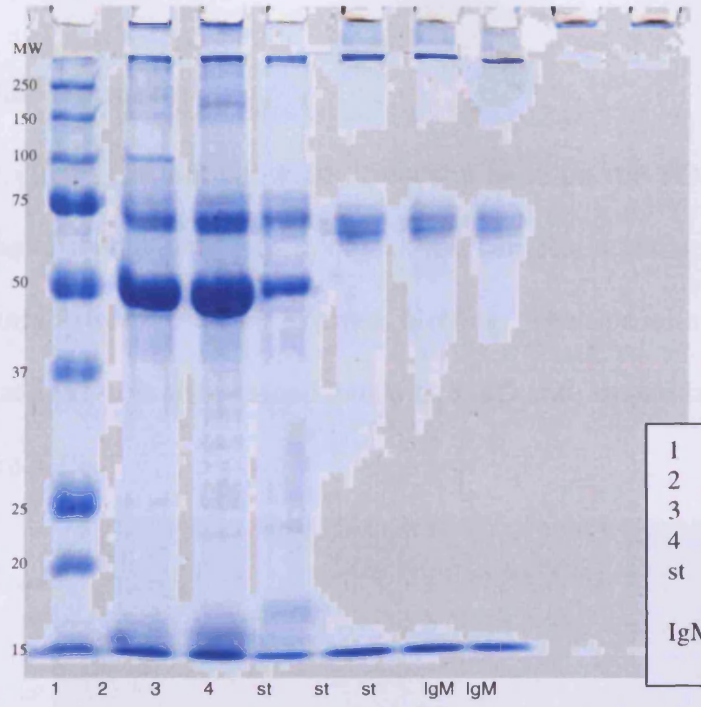
Figure 3.12 Western blot of serum albumin (left) and IgA (right). On each membrane, the first six lanes after the marker are tear samples and the last two lanes are protein standards. Each membrane shows that standard coincides with the required protein and the comparison of the membranes show that the proteins have different molecular weights and locations.

Tear samples from three subjects were used for the first six lanes and two standards for the last lanes (Figure 3.12). The applied volumes on the gel were equivalent to the volumes used for experiments with silver staining. The used IgA antibody was specific to the IgA α-chain that has a molecular weight of 75kD. This Western blotting showed that IgA stayed with its α-chain on the top of the resolving gel, as was expected since secretory IgA has a molecular weight of 400kD.

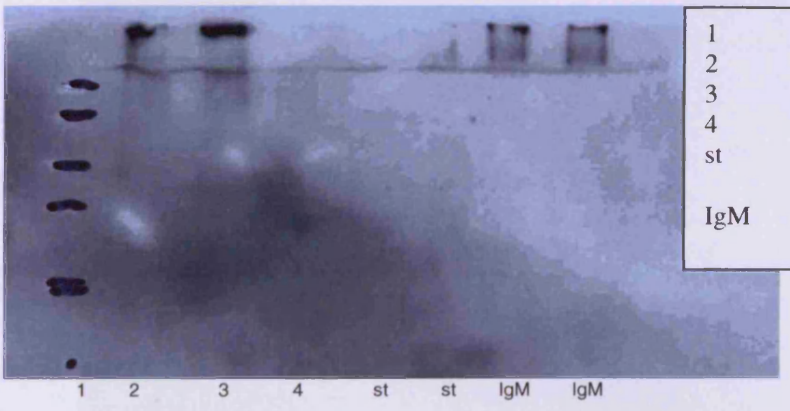
3.3.3.5 IgM

When infant tears were subjected to SDS-PAGE a faint band was observed on the top of the stacking gel (Figure 3.13). IgM has a molecular weight of 900kD and was suspected to be this protein band. Western blotting was used to examine this band.

Figure 3.13 SDS-PAGE of premature infant tears. Tears from three premature infants are seen on lanes 2-4 (Figure 14). Two of these infants (Premature A and Premature B) were twins and had a distinct band on the top of the stacking gel. The third infant whose tears were collected on the same day did not show this band on the SDS-PAGE.



1	Marker
2	premature infant PA
3	premature infant PB
4	premature infant PC
st	standard mix (IgA, lactoferrin, lysozyme)
IgM	IgM standard



1	Marker
2	premature infant PA
3	premature infant PB
4	premature infant PC
st	standard mix (IgA, lactoferrin, lysozyme)
IgM	IgM standard

Figure 3.14 Western blotting of premature infant tears with IgM antibody

Western blotting with an IgM antibody showed that all visible bands on the top of the stacking gel were IgM (Figure 3.14).

3.3.3.6 Lipocalin

Some infants did not show any lipocalin band on the SDS-Page. In order to verify that the observed band was lipocalin, tear samples remaining from premature and full-term infants were used for Western blotting. The lipocalin antibody stained the target band at 18kD. It also stained bands at 37kD and, in one lane, a band at about 250kD (Figure 3.15).

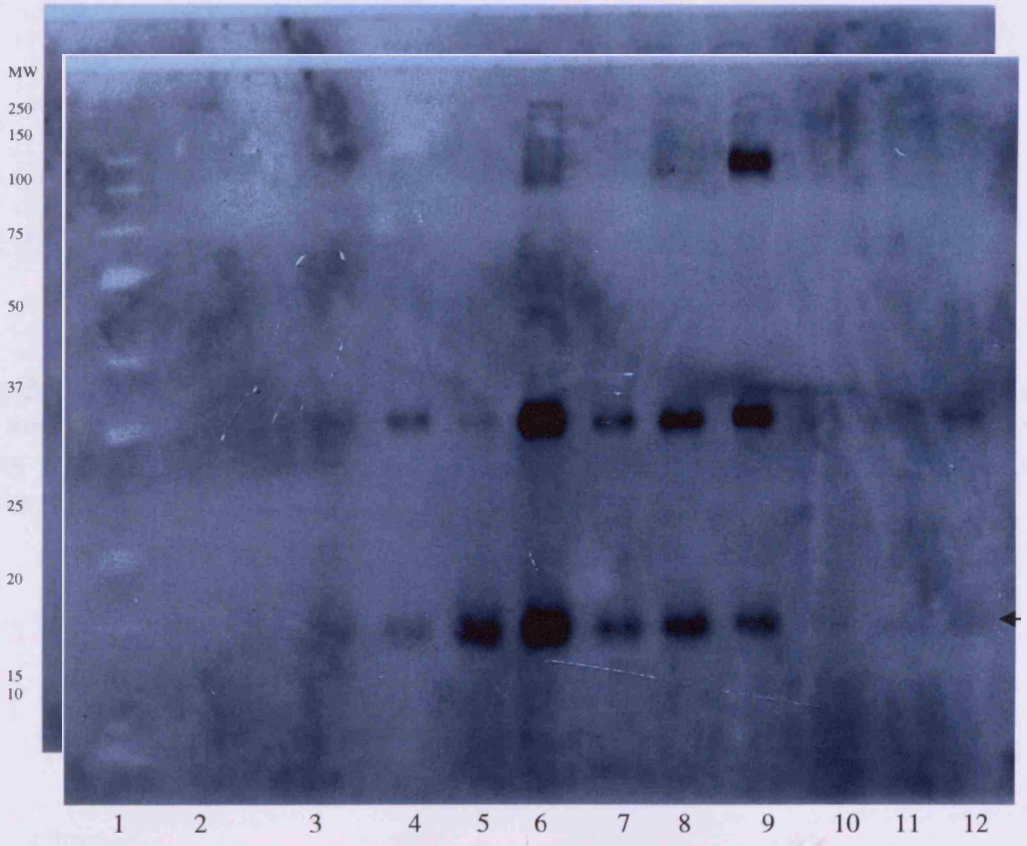


Figure 3.15 Western blotting of infant tears: Lane 1 contains the protein marker. Lanes 2-10 contain the tears of a different infant. The last two lanes (11 and 12) contain the standard mix (IgA, lactoferrin, and lysozyme). The arrow indicates the target protein lipocalin.

The non-specific binding was surprising since no band could be detected at 37kD when gels were observed (Figure 3.16). Unfortunately no lipocalin standard is currently available to control Western blotting.

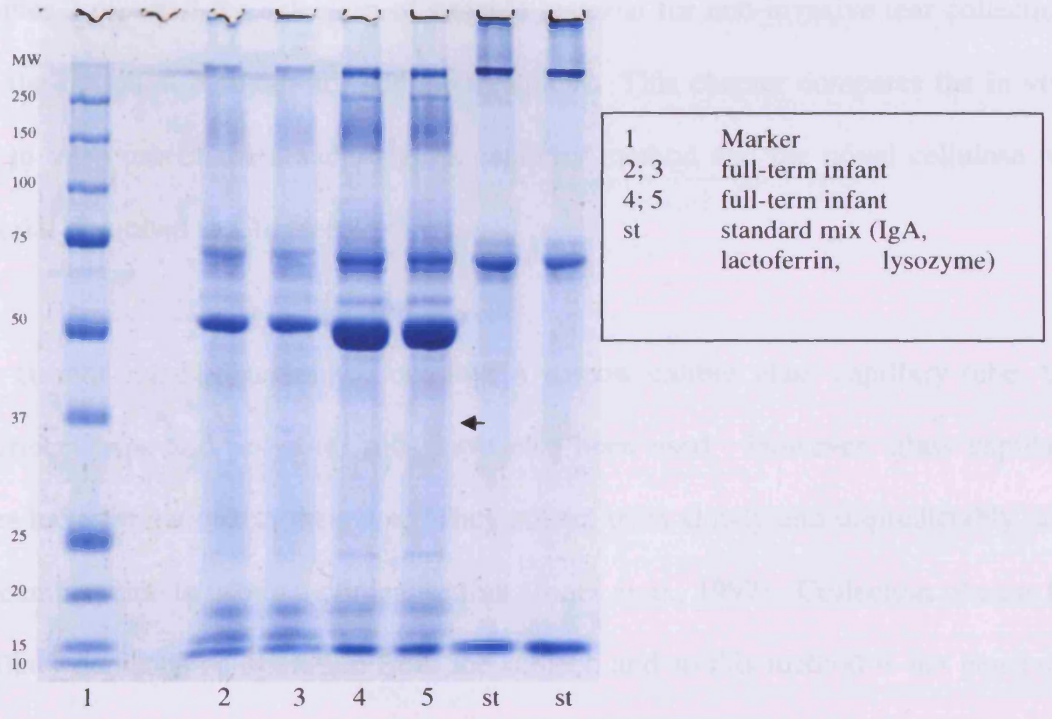


Figure 3.16 SDS-PAGE of two different infants: The lanes show lipocalin at 18kD and many different bands that are unknown. Although when Western blotting was performed the lipocalin antibody appeared also at 37kD, no band could be detected (arrow).

4 Tear sample collection using cellulose acetate absorbent filters

4.1 INTRODUCTION AND AIMS

Chapter 2 reported the selection of suitable material for non-invasive tear collection, and the optimum methods for sample treatment. This chapter compares the in vivo and in vitro use of the standard glass capillary method and the novel cellulose rod material described in Chapter 2.

The current standard technique employs a narrow calibre glass capillary tube, but Schirmer strips and polyester rods have also been used. However, glass capillary tubes have limitations in their use. They collect tears slowly and unpredictably, and are cumbersome to use in a clinical setting (Jones et al., 1997). Collection of tears by capillary demands co-operation from the subject, and so this method is not generally used for mass screening or tear collection from children (van Agtmaal et al., 1987; Norn, 1992). The alternative collection methods utilise absorbent materials, but cause ocular surface irritation. Collection papers and threads, such as Schirmer strip papers (Schirmer, 1903), are left to rest on the eye surface (Patrick, 1974) and may require the use of anaesthetics (Isenberg et al., 1998; Toker et al., 2002). The optimal safe tear collection technique requires a combination of rapid collection and control of reflex tearing, while allowing good extraction properties and efficient tear analysis after storage.

This chapter reports on a new tear collection method, using the filter rods of cellulose acetate. A series of studies is presented that compares the cellulose rod with the glass capillary tube:

- To show efficiency of tear collection with each collection technique (Experiment 1, 2, 3 and 4)).
- To investigate storage within the selected material (Experiment 1 and 2).
- To assess the safety of the technique for the ocular surface (Experiment 5).
- To assay protein content for both techniques (Experiment 6).

Prior to the experiments, the next section will present the tear collection methods used, their preparation and how tear collection was performed.

- Glass capillary tubes

Narrow bore glass capillary tubes (Sigma-Aldrich, UK) (Figure 4.1a) were obtained, and heat modified by stretching to produce a fine tip. The tubes were calibrated, by absorption of a fixed volume of distilled water, and marked for 2µl. For sample collection, the subject was asked to sit, with their head resting back against the seat headrest, and with an upward and slightly nasal gaze. The tube tip was held close to the lower lid, with the tip resting in the lower tear meniscus. The user was then able to observe the volume of tears transported up the capillary tube. After the sample was collected, the tube was sealed in an Eppendorf tube and placed on ice to preserve the sample until processing. The sample was then expelled into a 0.5ml Eppendorf tube and the extracted sample volume measured using a P2.5 or P10 micropipette (Biohit, UK).

- Cellulose rods

The cellulose rods (Filtrona Fibertec, Germany) (Figure 4.1b) were used as described in Chapter 2. Prior to use, the rods were sterilised under UV radiation for 5 minutes. Samples were extracted and tear volumes were measured by the optimum protocol in Chapter 2 by centrifugation. For sample collection, the subject was asked to take the same posture and gaze position as for the capillary tube, and the cellulose rod was held, with forceps, close to the lower lid, with the rod tip resting in the lower tear meniscus. The rod was held for a fixed period of time, with breaks every 5 seconds to allow blinking and avoid reflex tearing, after which it was sealed in a 0.2ml Eppendorf tube and placed on ice until sample processing. Collection time for each experiment was varied between 2-3 minutes and is noted in the "methods and materials" sections.

Figure 4.1a

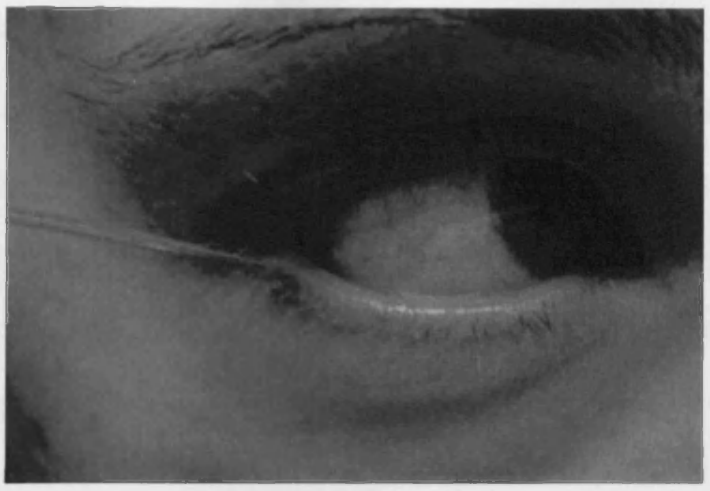


Figure 4.1b

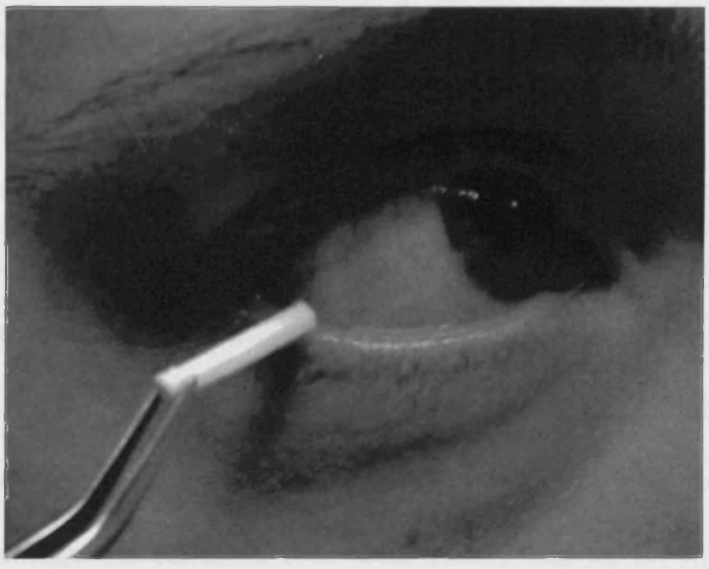


Figure 4.1 Tear collection with glass capillary tube (a) and cellulose rod (b)

4.2 EXPERIMENT 1: Tear collection efficiency in vitro

4.2.1 Introduction

The efficiency of the glass capillary tube (CT) and cellulose rod (CR) were tested *in vitro* by absorbing and releasing a sample. This sample contained a protein standard with known volume and concentration. Additionally the effect of freezing and storage on the protein standard was tested. This study will show any alterations to the sample that is caused by the collection material and the processing steps.

4.2.2 Methods and materials

5µl of bovine serum albumin (BSA) (2µg/µl) (Sigma-Aldrich, UK) protein standard was pipetted into the capillary tube or cellulose rod. The samples were extracted immediately and their volume and protein concentration assessed. This was repeated for 6 capillary tubes and 6 cellulose rods on 3 separate days. To assess the effect of storage, the collection procedure was repeated. The capillary tube samples were extracted immediately and stored along with the cellulose rods samples, for 7 days at -20°C. After storage the cellulose rods were gently thawed for 30 minutes before sample extraction by centrifugation, while the capillary tube samples were left thawed for the same amount of time. Each sample was analysed for volume and protein concentration. Once samples were extracted, the Eppendorf tubes were masked to prevent bias in analysis.

4.2.3 Results

All results were checked for normality, and if necessary were log-transformed, and the appropriate parametric were applied (ANOVA repeated measures, Friedman Test, SPSS 11, USA). Non-parametric tests were applied if log-transformation of the data was unsuccessful.

The mean sample volume and BSA concentration after extraction, for both techniques, are given in Table 4.1. For the capillary tube, there was a slight reduction in sample volume after extraction[1]. Freezing decreased the extracted volume further ($p=0.015$, Friedman Test). For the cellulose rod, the volume decreased from the initial standard volume[1], but there was no additional decrease in extracted sample volume after storage ($p=0.3$, Friedman Test).

For the capillary tube the BSA concentration was slightly reduced while the BSA concentration of the cellulose rods was increased after extraction[1]. For the capillary tube the protein concentration between fresh and stored samples was reduced, but not significantly different ($p=0.05$, ANOVA repeated measures). In contrast, the extracted BSA concentration from cellulose rods increased compared to the BSA standard concentration, but the difference between fresh and stored samples was not significant ($p=0.15$, ANOVA repeated measure).

Between the two methods, storage altered volume ($p<0.001$, Friedman test), but had no effect on the BSA concentration ($p=0.09$ ANOVA, repeated measure).

[1] statistical testing for differences is not suitable. The volume and concentration before the extraction are given and have no standard deviation

Table 4.1 Mean±sd sample volumes (µl), and BSA and tear protein concentrations (µg/µl), for the two techniques, from fresh and stored samples (n=18 for each of the results).

	Capillary Tube		*Cellulose Rod*	
	Fresh	*Stored*	*Fresh*	*Stored*
BSA Volume (5µl)	4.9±0.2	4.5±0.9	3.7±0.3	3.9±0.3
BSA Standard (2µg/µl)	1.81±0.18	1.64±0.31	2.54±0.51	2.17±0.55

4.2.4 Conclusion

For the *in vitro* study, the BSA concentration after extraction from cellulose rods exceeds the initial concentration while sample volume is decreased. The increase in BSA concentration must be due to the retention of liquid within the rod, or to evaporation of the sample from the rod. For the capillary tube the volume is not altered after extraction, but the BSA concentration was decreased. These effects have to be taken into account when differences in protein concentrations between both methods are considered. This experiment also shows that storage within the cellulose rod is well tolerated for both volume and protein content.

4.3 EXPERIMENT 2: Tear collection efficiency in vivo

4.3.1 Introduction

To test the efficiency of both collection techniques and the effect of freezing and storage *in vivo*, tears were repeatedly collected from the same subjects on different days.

4.3.2 Methods and materials

Seven subjects (3 female, 4 male; mean±sd age 26.3±3 years; range 19-31) were recruited. All subjects were members of staff and students of Cardiff University. Subjects were excluded for systemic or ocular surface disease, medication known to affect the ocular surface, contact lens wear, allergies, pregnancy and dry eye. Tear collections were completed before noon to avoid fatigue from computer use or laboratory work, and to reduce the effect of any diurnal variation.

Local ethical committee approval was obtained and informed consent was obtained from all subjects prior to participation. The study protocols complied with the Declaration of Helsinki.

Two non-stimulated tear samples were taken from each subject, one with a capillary tube and one with a cellulose rod, in a random order. Collection time was limited to 3 minutes and a 30 minute interval was allowed between collections. Once both samples were extracted, the Eppendorf tubes were masked to prevent bias in analysis. This was repeated on 3 separate days, and samples were analysed for volume and total tear protein concentration. The collections were further repeated to assess the effect of storage at -20°C, using the same protocol stated previously.

4.3.3 Results

All results were checked for normality, if necessary log-transformed and the appropriate parametric and non-parametric tests were applied (ANOVA, repeated measure and Wilcoxon Signed Rank test, SPSS 11, USA).

The cellulose rod technique collected a significantly larger sample volume than the capillary tube, for both fresh and stored samples (p=0.003 all samples, p=0.04 fresh samples, p=0.06 stored samples, Wilcoxon Signed Rank test[2]) (Table 4.2). Storage had no effect on volume collected, (p=0.85 for all samples, p=0.11 for CT, p=0.49 for CR, Wilcoxon Signed Rank test). There was no significant difference between techniques for total tear protein concentration (p=0.17 ANOVA, repeated measure).

Table 4.2 Mean±sd sample volumes (µl), and BSA and tear protein concentrations (µg/µl), for the two techniques, from fresh and stored samples (n=21 for each of the presented results).

	Capillary Tube		*Cellulose Rod*	
	Fresh	*Stored*	*Fresh*	*Stored*
Tear Volume (µl)	2.7±1.3	4.1±1.4	7.6±7.4	5.3±5.4
Tear Protein Concentration (µg/µl)	11.21±3.86	11.9±2.77	13.87±3.12	12.27±2.27

4.3.4 Conclusion

The *in vivo* study highlights the effectiveness of the cellulose rod over the capillary tube in collecting large tear samples. The effect on protein concentration shown in Experiment 1 is borne out by the *in vivo* study where the tear protein concentration extracted from the cellulose rods was higher than the capillary tube samples, although not significantly.

From a practical point of view, when tears are collected, storage of the samples for later analysis is frequently needed. The results in both Experiments 1 and 2 indicate that storage at –20°C can be successfully used to preserve the tear samples prior to

[2] The results for volume size showed a day to day variation (p=0.04, ANOVA repeated measure) for each subject and were treated unpaired.

extraction, for at least one week after collection. This supports Van Agtmaal et al. (1987) who recommend storage at -20°C.

When tears were collected by the cellulose rod, it was observed that three extracted samples from the cellulose rod had a low volume of 0.5µl and one sample had no volume. Experiment 1 showed that 68% to 80% of the BSA standard can be recovered. In balance then, the deficiency of the rod in releasing the sample is more than compensated for by the efficiency of collection, and has no detrimental effect on tear component analysis. The next experiment was conducted to examine the volumes that can be obtained by extraction of small sample volumes.

4.4 EXPERIMENT 3: The effect of sample size on the extracted volume

The effect of initial sample size on the extracted volume was assessed by decreasing the volume size of the solution aspired into the cellulose rods.

4.4.1 Methods and materials

Different initial volumes (0.3, 0.6, 0.9, 1.5, 5 and 10µl) of 2% saline (Sensitive Eyes™ Plus Saline, Bausch and Lomb, UK) were pipetted into the cellulose rod, and then immediately extracted to calculate the percentage of sample extracted.

4.4.2 Results

Table 4.3 shows the percentage of volume extracted compared with volume inserted. Results were graphically plotted to find best fitting line (Microsoft Excel 2002, USA). The larger the sample volume, the larger the percentage of sample extracted (logarithmic, r=0.88 p=0.02).

Volume Inserted (µl)	Volume extracted (µl)	Percentage Extracted (%)
0.3	0	0
0.6	0.23±0.12	39
0.9	0.43±0.14	48
1.5	1.07±0.2	71
5	3.38±0.56	68
10	7.86±0.38	79

Table 4.3 Inserted and extracted sample volume (µl) and the percentage extracted from the cellulose rod

4.4.3 Conclusion

In the sample collection, extraction and storage studies reported, the cellulose rod was shown to be able to absorb a wide range of tear volumes in very short time. However, while the high absorbency of the cellulose allowed a rapid and efficient collection, it hindered the full release of the sample during extraction. Preliminary studies (Chapter 2) assessed the optimum parameters for extraction using centrifugation and found that, with any of the examined materials the volume returned was always less than that absorbed. Some proportion of the sample is always retained within the cellulose

rod or evaporates during usage of the rod. This effect is greater for smaller sample sizes of less than 0.6µl.

4.5 EXPERIMENT 4: Reflex tearing and tear collection

4.5.1 Introduction

To assess any relationship between sample volume and total tear protein concentration.

4.5.2 Methods and materials

Six subjects (3 female, 3 male, mean±sd age 23.2±4.5 years, range 19-31) were recruited (subject recruitment as described in Experiment 2). Two tear samples were collected from each subject on 2 different days, over 2 minutes, with 30 minutes between each sampling. On each day only one method was used, the first sample was non-stimulated, and the second sample was stimulated by nasal stimulation. All samples were extracted and processed immediately for volume and total tear protein concentration.

4.5.3 Results

All results were checked for normality and if necessary log-transformed. Thereafter appropriate parametric tests (ANOVA, repeated measure and Pearson's Correlation Coefficient) were used (SPSS 11, USA).

Significantly higher sample volumes were collected with the cellulose rod, for both stimulated and non-stimulated samples (p=0.001, ANOVA repeated measure after log-transfer) (Table 4.4).

Table 4.4 Mean±sd sample volumes (µl) and tear protein concentrations (µg/µl), for the two techniques, with and without stimulation.

	Capillary Tube		Cellulose Rod	
	non stimulated	stimulated	non-stimulated	stimulated
tear protein concentration (µg/µl)	13.9±3.2	11.3±2.5	13.2±4	9.6±1.8
tear volume (µl)	1.3±1	2.5±1.4	10.5±9.7	30.1±22.4
pearson correlation (r)	-0.56 (p=0.074)		-0.66 (p=0.011)	

However, there was no significant difference in total tear protein concentration between techniques or stimulated/non-stimulated samples. Pearson's correlations were used to investigate the relationship between total tear protein concentration and sample volume (Figure 4.2). No significant correlation was found for the capillary tube although the small sample volumes collected limit the analysis. In contrast, the cellulose rod was able to collect much larger sample volumes, and the graph is able to describe a gradual reduction in protein concentration with increasing sample volume following nasal stimulation.

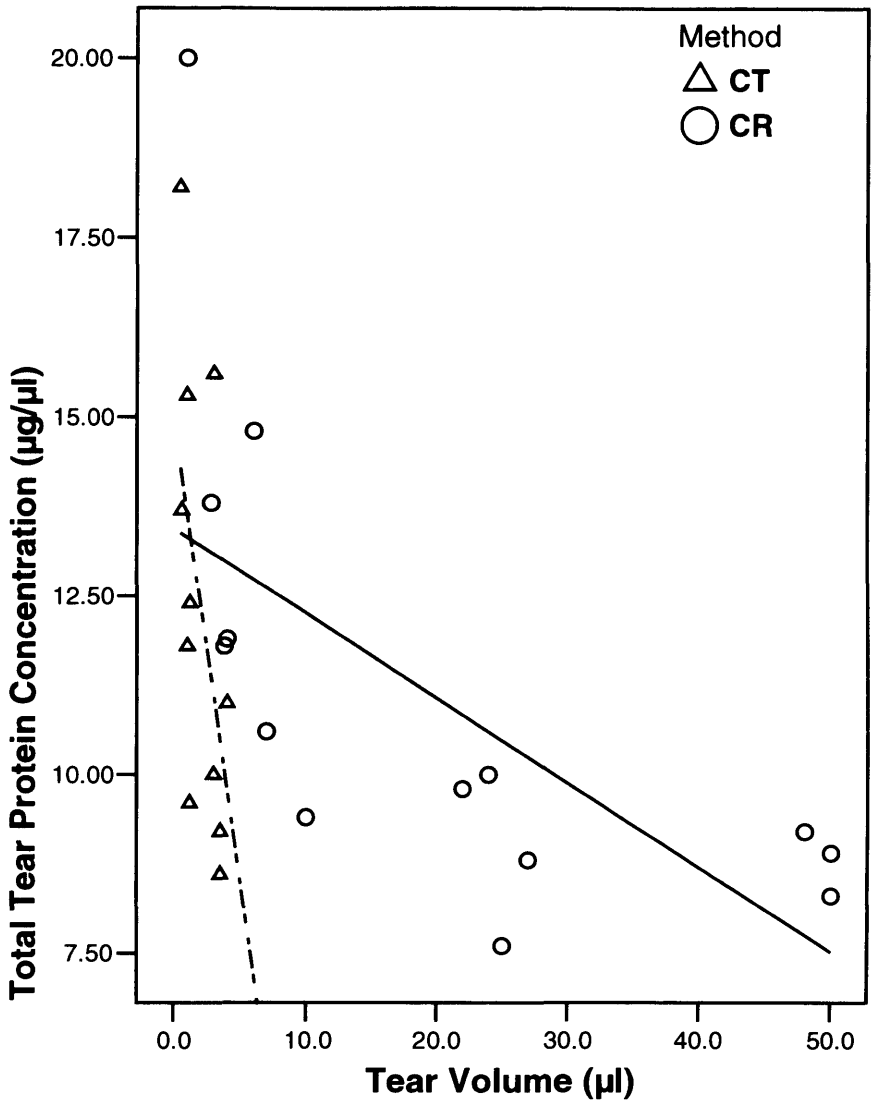


Figure 4.2 Relationship between total protein concentration and sample size: The trend lines (dashed for CT and solid for CR) show the reduction in the protein concentration with increase of tear secretion.

4.5.4 Conclusion

Stimulation of tear production naturally caused larger collected samples for both collection techniques, but the absorbency of the cellulose rod was better able to respond to the increased tear availability. This emphasises the limitation of the capillary tube in quickly collecting any available tears.

The next experiment examined the invasiveness of the cellulose rod compared to the capillary tube.

4.6 EXPERIMENT 5: Technique invasiveness

4.6.1 Introduction

Measurement of the serum albumin concentration in the tear sample was used to assess the level of technique invasiveness. Serum albumin can leak from the blood vessels of the bulbar conjunctiva if the collection material is pressed against the ocular surface (Stuchell et al., 1984). In contrast, touching the eyelid or lashes may produce reflex tearing, reducing serum albumin concentration. This experiment aimed to assess these effects.

4.6.2 Methods and materials

27 subjects (17 female, 10 male; mean±sd age 27.4±4.6 years; range 22-35) were recruited. Recruitment is described in Experiment 2. One tear sample was taken with each technique, in a random order, over a maximum time period of 1 minute 45 seconds. A 30 minute interval was allowed between collections. With the capillary tube, sample collection was halted once a minimum sample size of 2µl was collected. This limitation was needed to produce standard volumes and served to avoid variation by dilution in the tear samples from the capillary tubes. Serum albumin and total tear protein concentrations were then determined with gel electrophoresis.

4.6.2.1 Gel Electrophoresis

Tear proteins were analysed following SDS-PAGE, as described in Chapter 3. For determining the serum albumin concentration, 0.25µl of non-denatured samples, human serum albumin standard (0.25µg/µl) (Sigma-Aldrich Co, UK) and 10µl pre-stained blue marker (250 and 10kDa, Precision Plus Protein Standard, Bio-Rad Laboratories, UK) were run on SDS-PAGE, visualised with silver stain (Pierce Biotechnology, USA). Scanning densitometry was performed to determine the intensities of serum albumin and standard bands. For analysis the ratio between these band intensities was used. A standard curve was established by gradual dilutions of the serum albumin standard.

4.6.2.2 Total Tear Protein Concentration Analysis

Remaining samples were assayed for total tear protein concentration using the method based on the Bradford test, described in Chapter 3.

4.6.3 Results

Of the 27 subjects recruited, 22 had sufficient sample volume (>0.5µl) with both techniques to permit a paired comparison of serum albumin concentration (Table 4.5). One of the samples with a low volume size was collected with a capillary tube. From four cellulose rods no tears were extracted. Depending on the sample volume 12 pairs of results were obtained for the total protein concentration.

Table 4.5 Mean±sd concentrations (μg/μl) of extracted sample volumes, serum albumin (SA) and total protein concentrations.

	Volume (μl) (n=27)	Ratio of SA and SA standard (n=21)	Total Protein (n=12)
capillary tube	1.5±0.6	0.3±0.16	7.6±4.3
cellulose rod	4.4±7.1	0.45±0.19	10.3±3.3
significance		p=0.003	p=0.08

When the ratios were compared to a standard curve, it was shown that all the concentrations were at the lower end of the range where the curve is linear. The highest possible concentration found with the cellulose rod was below 0.1μg/μl. Therefore the results shown in Table 4.5 are ratios between the serum albumin band in the tear sample and the standard. The normality of the serum albumin results was tested and the difference between the ratios was compared by paired t-test (SPSS 11, USA). The cellulose rod samples had significantly higher concentrations of SA, although still at a very low level. For the cellulose rod, SA concentration was also compared with tear volume, and a strong negative correlation found (r=-0.62, p=0.002) (Figure 4.3). This reduction in protein concentration with increasing sample volume is similar to that shown in Figure 4.2.

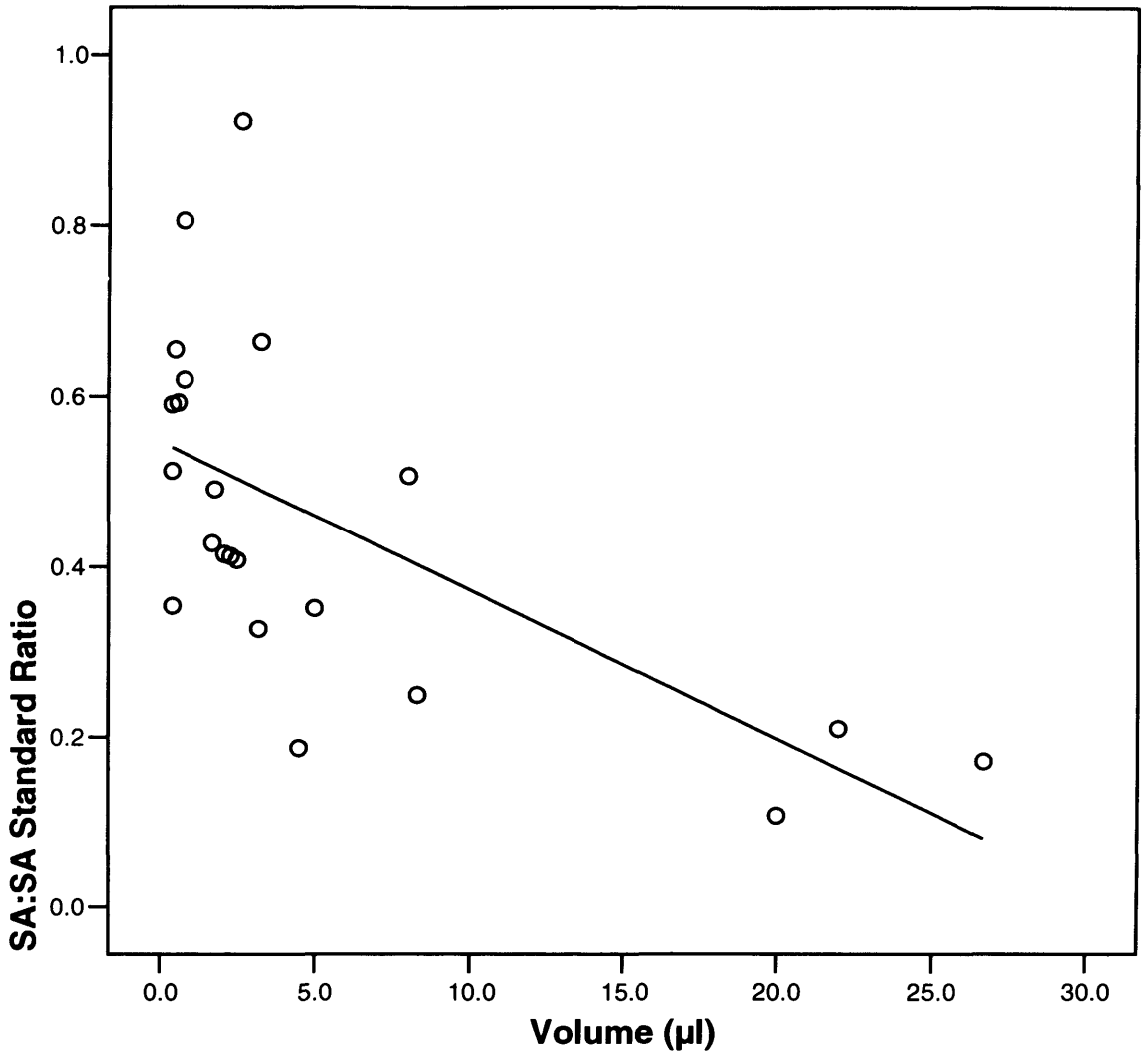


Figure 4.3 Correlation between the tear SA concentration and increasing sample size found for the cellulose rod

4.6.4 Conclusion

The difference found in the protein concentration of both techniques may be a result of the same effects seen in Experiment 1. The cellulose rod is capable of collecting tear volumes with minimal invasiveness. If tears are stimulated no serum leakage is found and serum albumin concentration does not increase.

4.7 EXPERIMENT 6: Major tear protein concentration

4.7.1 Introduction

The two techniques were compared in their ability to collect and release the major tear proteins.

4.7.2 Methods and materials

Sixteen subjects (7 female, 9 male; mean±sd age 25.6±3.3 years; range 22-31) were recruited. Recruitment is described in Experiment 2. Non-stimulated tear samples were collected with each technique in a random order. Collection time was limited to 2 minutes. The samples were processed immediately, and the major protein concentrations determined as described in Chapter 3.

For major protein determination, 0.5µl of non-denatured sample, known protein standards (human sIgA; 1.8µg/µl, human milk lactoferrin; 2.5µg/µl, hen egg lysozyme; 2.5µg/µl, Sigma-Aldrich, UK), and 10µl pre-stained blue marker (250 and 10kDa, Precision Plus Protein Standard, Bio-Rad Laboratories, UK) were loaded on appropriate gels. The protein bands were visualised by blue staining (GelCode® Blue Stain Reagent, Pierce Biotechnology, USA) and concentrations were determined by comparing band intensities to the protein standards'.

4.7.3 Results

Results were tested for normality and appropriate tests were used (SPSS 12, USA). Tears were successfully extracted from 14 cellulose rods. Two sample,(one sample

for each collection method) were too low in volume (<1μl) to be subjected to the SDS-PAGE. Significantly higher sample volumes were collected with the cellulose rod than the capillary tube (p=0.012, unpaired t-test, equal variances not assumed) (Table 4.6). The paired t-test was significant (p=0.014), but the value of the correlation coefficient and the significance value for each pair of variables used in the paired samples t-test procedure was low (r=0.37, p=0.2), so that the samples can be treated as independent samples.

Table 4.6 Mean±sd concentrations (μg/μl) of the major tear proteins and extracted sample volumes.

	Volume (μl) (n=16)	IgA (n=12)	Lactoferrin (n=12)	Lysozyme (n=12)
capillary tube	**2±0.6**	**1.77±0.79**	**2.07±0.87**	**2.61±1.36**
cellulose rod	7.8±8.4	1.91±0.87	2.26±0.99	2.52±1.7
significance	**p=0.012**	**p=0.5**	**p=0.2**	**p=0.7**

No significant differences were found for the major tear protein concentrations (Table 4.6, paired t-test).

4.7.4 Conclusion

To assess the cellulose rod, in its ability to not alter the tear sample composition, and the concentration of some major tear proteins were measured after extraction. No significant differences were found between the two collection methods.

4.8 DISCUSSION

This study has shown that the cellulose rod can be successfully used as an alternative method for tear collection to the glass capillary tube. It is able to quickly absorb a sample, while being minimally invasive. It provokes similar, or less, reflex tearing to the capillary tube, without causing any detectable trauma. The sample can be quickly extracted when needed, can be stored within the rod for short periods, while frozen, without any detrimental effect to the sample, and the cellulose rod does not appear to affect the analysis of the tear components, such as the protein concentration assay.

For analysis purposes, large tear volumes are usually collected in tear film studies (Glasgow et al., 1999; Choy et al., 2004; Dogru et al., 2004). Studies (White et al., 1993; Fukuda et al., 1996; Aho, Nevalainen and Saari, 2002; Choy et al., 2003) that compare reflex to non-stimulated tears show alteration to the tear film from its normal state. Since the non-stimulated tear volume is very small, and the tear flow rate is around 1.2µl/minute (Mishima et al., 1966), collecting a sufficiently large sample requires controlled collection of small volumes (Fullard, 1988) or it requires a very long time (Fullard and Snyder, 1990). The glass capillary tube has long been the standard method for tear collection, but it collects slowly, is known to induce reflex tearing, and requires a co-operative subject. For some situations, such as tear collection in infants, the use of the glass capillary tube will cause ethical problems. Of the samples collected with the cellulose rod in these studies, half had a volume more than 2µl, which were collected in a patient-friendly time of less than 1 minute 45 seconds. Although a small proportion of the sample was not recovered due to absorption or evaporation, this effect did not significantly affect the analysis.

It was shown that some samples collected with the cellulose rod may be diluted. This dilution effect was minimal, so that no difference was found between the protein concentrations of both methods. Previous studies (Coyle and Sibony, 1986; Fullard, 1988; Ng et al., 2000) with the capillary tube that used the Bradford test for total tear protein determination, compare well with the results in this study and confirm that larger sample sizes have lower total tear protein concentrations (Table 4.7).

Table 4.7 Comparison with previously reported sample volumes and total tear protein concentrations.

Sample Volume	Collection method	Total tear protein (mean±sd µg/µl)	Reference
10-120µl	CT (non-stimulated)	7.1±1.9	Coyle and Sibony (1986)
Minimum of 70µl	CT (yawn reflex)	6.05±1.58	Ng et al. (2000)
50-300µl	CT (nasal stimulation)	6.0	Fullard (1988)
0-5µl	CT (nasal stimulation)	9.1	
2µl	CT (non-stimulated)	17.2	
7.8±8.4 (mean±sd µl)	CR (non-stimulated)	13.87±3.12	Current study
2±0.6 (mean±sd µl)	CT (non-stimulated)	11.9 ±2.8	

The specific major tear proteins assayed were from the lacrimal gland and were selected for their significance in tears: lactoferrin and lysozyme concentration is identical in stimulated and non-stimulated tears (Fullard and Tucker, 1991). While awake, lysozyme makes up almost half of the proteins in tears. IgA is under a

different secretion control (Dartt, 1989) and during sleep it becomes more dominant (Sitaramamma, Shivaji and Rao, 1998b). The lactoferrin assay has been proposed as an important test for diagnosing keratoconjunctivitis sicca (Danjo et al., 1994; McCollum et al., 1994; Da Dalt et al., 1996; Wang et al., 2005).

No difference was found in concentrations for these major tear proteins between the two techniques. Furthermore, apart from IgA, which was higher compared to other studies (Sen and Sarin, 1979; Gachon, Richard and Dastugue, 1982; McGill et al., 1984), the concentrations were similar to the results from previous studies for lactoferrin (Kijlstra et al., 1983; Berta, 1986; Fullard and Tucker, 1991; McCollum et al., 1994; Ng et al., 2000; Ohashi et al., 2003) and lysozyme (McGill et al., 1984; Berta, 1986; Sen and Sarin, 1986; Fullard and Tucker, 1991; Sitaramamma et al., 1998a; Ng et al., 2000) (Table 4.8). Since IgA secretion is regulated differently to lysozyme and lactoferrin (Dartt, 1989), the high IgA concentration may be due to the avoidance of reflex tearing. The increased IgA may be due to poor band differentiation during analysis (Kuizenga et al., 1991). However, more significantly for this comparison, there was no difference in IgA concentration between the two collection techniques.

Table 4.8 Comparison with previously reported major tear protein concentrations.

References: IgA	Collection method	Protein analysis	Total tear protein (mean±sd µg/µl)
Sen and Sarin (1979)	CT (non-stimulated)	Immunodiffusion	0.24±0.15
Gachon et al. (1982)	CT (non-stimulated)	Immunological	0.41±0.15
McGill et al. (1984)	filter paper	ELISA	0.52±0.14
Current study	CR (non-stimulated)	SDS-PAGE	1.91±0.87
	CT (non-stimulated)		1.77±0.79
Lactoferrin			
Kijlstra et al. (1989)	CT(nasal stimulation)	SDS-PAGE	2.22±1.21
Berta (1986)	CT(nasal stimulation)	SDS-PAGE	2.24±0.84
Fullard and Tucker (1991)	CT (non-stimulated)	HPLC/ELISA	1.65±0.15
McCullum et al. (1994)	CT(traumatic stimulation)	LactoCard	1.48
Ng et al. (2000)	CT(yawn reflex)	SDS-PAGE	2.73±0.82
Ohashi et al.(2003)	CT (non-stimulated)	ELISA	2.05±1.12
Current study	CR (non-stimulated)	SDS-PAGE	2.26±0.99
	CT (non-stimulated)		2.07±0.87
Serum albumin			
Ng et al. (2000)	CT(yawn reflex)	SDS-PAGE	0.021±0.028
Fullard and Tucker (1991)	CT(non-stimulted)	HPLC/ELISA	0.042±0.005
Stuchell et al. (1984)	CT(non-stimulated)	Immunological	0.018±0.06
	Schirmer paper		1.24±1.42
Lysozyme			
McGill et al. (1984)	filter paper	ELISA	1.10±0.22
Sen and Sarin (1986)	CT (non-stimulated)	Immunodiffusion	1.31±0.49
Berta (1986)	CT(nasal stimulation)	SDS-PAGE	2.01±0.62
Fullard and Tucker (1991)	CT(non-stimulted)	HPLC/ELISA	2.07±0.24
Ng et al. (2000)	CT(yawn reflex)	SDS-PAGE	2.46±0.44
Current study	CR (non-stimulated)	SDS-PAGE	2.49±1.7
	CT (non-stimulated)		2.61±1.36

Serum albumin quantification was used to assess the invasive nature of the two techniques on the conjunctiva and cornea. Invasive tear collection can result in serum albumin (SA) leakage from the conjunctiva (Josephson and Lockwood, 1964). Stuchell et al. (Stuchell et al., 1984) measured 0.018±0.06 (μg/μl) SA in CT samples, with an increase to 1.24±1.42 (μg/μl) when samples were collected by Schirmer paper. The SA concentrations found in this study were low and within the range found by other investigators (Fullard and Tucker, 1991; Ng et al., 2000). The results show a general effect of a reduction in concentration with higher volumes, with any high individual SA concentrations well below any clinical significance. In other words, the statistically significant increase in SA concentration that was found in samples from the cellulose rods was not clinically significant.

This is not the first study to consider an absorbent material for tear collection. Porous polyester rods were used by several investigators (Jones et al., 1997; Afonso et al., 1999; Solomon et al., 2001) to collect tears for analysis of high and low abundance proteins. However, the polyester rod was rejected when it was found to release an unknown contaminant that caused an apparent increase in BSA concentration (Chapter 2). A recent study used polyurethane minisponges that were placed on the lower lid margin and left for 5 minutes (Lopez-Cisternas et al., 2006). A flaw in the methodology of this study was the denaturation of the sample that prevents the measurement of IgA and lactoferrin concentrations. However, after successive tear collection, a strong serum albumin band appeared on the gels that was strongly stained with the less sensitive coomassie blue stain. This shows that the tear collection was highly invasive and altered the protein profile.

Tuft and Dart (Tuft and Dart, 1989) compared IgE protein recovery and absorption effects between cellulose sponges and glass capillary tubes. They found no significant difference between the techniques, but they determined that the cellulose sponge was less time consuming, and did not influence tear analysis. Van Agtmaal et al. (van Agtmaal et al., 1987) also assessed a cellulose sponge's volume efficiency and storage effects. They found that it could absorb 6x more volume than Schirmer papers, with absorption and evaporation effects likely to be less with cellulose sponges.

Schirmer papers have also been in use, but the irritation they cause affects the significance of any research finding (Craig and Blades, 1999). Schirmer papers are often used with anaesthetics to avoid reflex tearing. Tears collected in this way, such as in neonates, are declared to be basal tears (Spiegler and Mayer, 1993; Akar et al., 2004). However, it is not clear if non-stimulated basal tears exist, and what the tears collected in this way represent (Jordan and Baum, 1980). No relationship has been shown between sample volume and tear flow under anaesthetics (Lamberts et al., 1979; Clinch et al., 1983).

In summary, these studies have shown the cellulose rod to be a safe, rapid and easy method for collecting an appropriate tear sample for protein assay. It exceeds the performance of the glass capillary tube in terms of ease and speed of sample collection, and does not alter the composition of the major tear components when extracted. In addition to its ease of use and speed of collection, the cellulose rod has the advantage of not presenting the same risk of trauma to the eye as the glass

capillary tube. This makes the technique particularly suitable for situations where the subject may be uncooperative, such as with infants and neonates.

5 Tear film collection and protein analysis from premature and full-term infants

5.1 INTRODUCTION AND AIMS

Having found and tested a suitable and safe collection technique, the cellulose rod, on adult tears, this chapter will move on to assess its use for full-term neonates, full-term infants and premature infants. The tear film quality of neonates has been found to be excellent, in particular the lipid layer, (Table 5.1) and this study will investigate the aqueous layer, which supports the lipid layer.

Table 5.1 Latest findings that acknowledge the excellent quality of the neonate tear film

Finding	Reference
Lower blink rate (2-3 blinks per minute)	(Lawrenson et al., 2003)
Long tear break-up time (NIBUT: 32.5+/- 5.2 seconds (range: 17.6-48.5 seconds)	(Isenberg et al., 2003)
Thickest tear lipid classification in 83.3% of all infants	(Isenberg et al., 2003)
Superior biophysical properties in the lipid layer of their youngest experimental group (1.5 years old)	(Kaercher et al., 1994)

In this chapter the collection method is applied to different groups of infants and adults. The first group were premature infants that were old enough to undergo the process of tear collection (P1). Some of them were available for a second collection (P2). The second group were newborn infants (F1). All of them were born full-term. In the third group were full-term infants with an age range between one week and twenty weeks (F2). Finally these groups were compared to a healthy adult group (A). The results were analysed together over a wide range of parameters, such as different maturity factors, gender differences and state of alertness.

Using this grouping, the tears were analysed as outlined in Chapter 3. The aims of this major study were:

- To confirm the suitability of the collection method to various classes of infants and to verify if the results with this technique compare to adults, the following factors were evaluated:
    - The distribution of tear volume in each group
    - The sensitivity of the technique for measuring the protein content and concentration

- To gain general knowledge about infant secretion, appropriate protein assays were used to examine these factors:
    - Tear protein profile, major proteins and other identified proteins and any relationship with maturity (age and weight variables), gender, alertness
    - The effect of known parameters, such as increased tear secretion

5.2 METHOD AND MATERIALS

5.2.1 Ethical approval and recruitment

All subjects were recruited by Dr. Patrick Watts (Department of Ophthalmology), and Dr. Patrick Cartlidge (Department of Paediatrics), at the University Hospital of Wales, Cardiff. Recruitment of subjects began as soon as ethical approval was obtained from the Central Office for Research Ethics Committees (COREC) and from the local NHS Research and Development Office (R&D) in Cardiff and Vale (Reference number 04/WSE02/125; date: 8/11/2004).

As part of the recruitment process, all parents received an information sheet about the study. Details of tear collection were discussed with them and an opportunity given for questions before written consent was obtained. Tears from infants were collected with the parent's assistance, or if parents were not present, a nurse's assistance. The assisting person was asked to stabilise the baby's head.

5.2.2 Subject groups

This section will describe the included subjects and their grouping.

5.2.2.1 Infant subject inclusion

For inclusion it was decided that only healthy subjects were to be recruited. Therefore the neonatalogist or the ophthalmologist and infant's records chart were consulted. Infants with Apgar scores within normal limits and without any abnormal systemic conditions were recruited. The Apgar score is a grading (0-10) of muscle tone, pulse,

reflex irritability, skin colour and respiration at one and five minutes after birth. A grading between 7 and the maximum of 10 is considered normal (Snow, 1998).

At first, the ocular surface was examined for signs of redness before and after tear sampling (increased blood flow in conjunctival vessels). The anterior segment was also examined for signs of epiphora, or conjunctivitis. If necessary, before collection, the infant was referred to the ophthalmologist (Dr. Watts).

Premature (P1) and full-term healthy newborn (F1) infants were recruited to form two subject experimental groups. Information about child maturation, such as gestational age (GA) and post-conceptional age (PCA), birth weight (BW) and actual weight, was obtained from parents or, if not available, from the infant charts. Infants that were seen a second time, were grouped separately (P2). Another group of full-term infants were recruited (F2) that were older full-term infants. The fifth experimental group consisted of adults (A). Table 5.2 shows the maturity factors (factors for age and weight at birth and at the time of the tear collection), gender and number of subjects recruited.

Table 5.2 Mean±sd gestational age (GA), post-conceptional age (PCA), post-natal age, birth weight (BW), weight on the day of tear collection (actual weight) and gender for each infant subject group

	Gender	GA (weeks)	PCA (weeks)	Age difference	BW (kg)	actual weight (kg)	Number of recruited infants
Premature (P1)	14 female, 16 male	29.9± 2.76	35.77± 1.77	2 weeks and 5days (± 6 days)	1.19± 0.48	1.87± 0.54	30
Premature (P2)	7 female, 5 male	28.63± 2.84	37.19± 1.76		1.06± 0.45	1.97± 0.43	12
Full-term newborn (F1)	19 female, 21 male	39.71± 1.27	39.86± 1.27	7 weeks and 4 days (± 6 days)	3.42± 0.53	no information available	40
Full-term (F2)	8 female, 6 male	40.04± 1.5	47.76±6.14		3.49± 0.48	5.04±1.34kg	14
Adults (A)	10 female, 12 male		24.95±3.63 years				22

If infants were asleep care was taken not to wake them up. This was an attempt to experience if the cellulose rods can collect tears with minimal invasiveness and disturbance.

During collection the head was held in a comfortable position (Figure 5.1). If possible the attention of the alert infant was directed towards a sound or voice, while the cellulose rod approached from another direction away from the cornea.

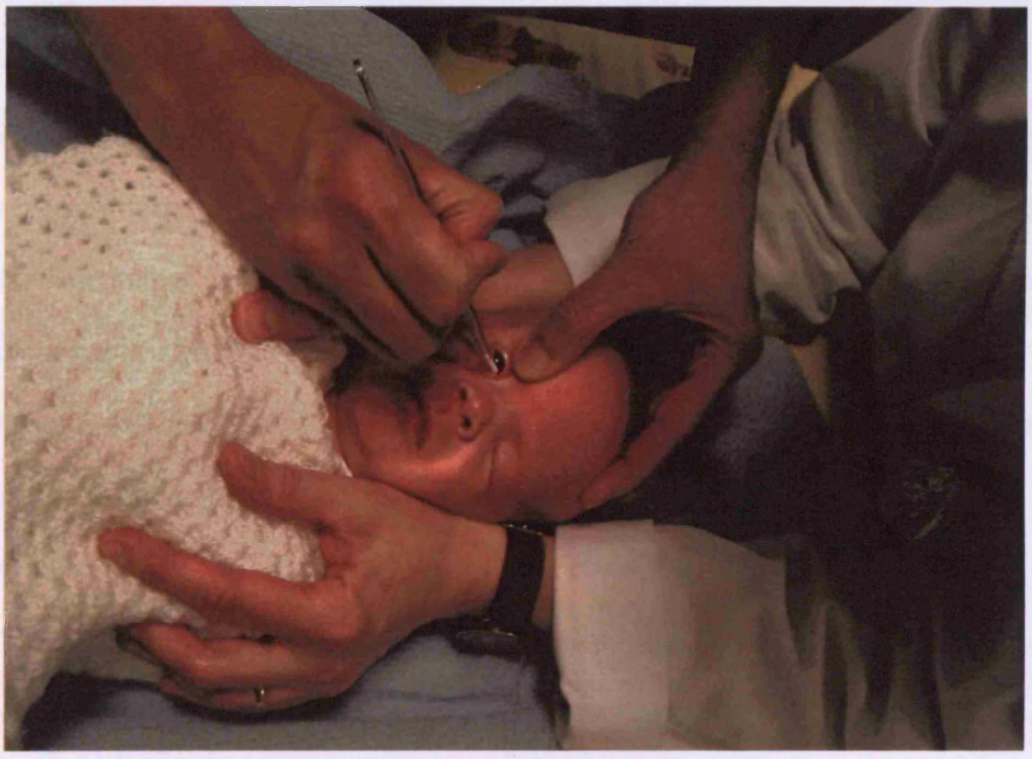


Figure 5.1 Tear collection from a sleeping premature infant with a cellulose rod (held with forceps)

For all infants the observed state of alertness was assessed in three categories:

- Closed eye: the eyes were closed for most of the time before and during tear collection
- Open eye: the infant was active and eyes were open
- Crying

5.2.2.2 Premature infants (P1 and P2)

Premature infants were investigated in Special Care Unit of the Univerity Hospital Wales, Cardiff. Some infants stayed in the hospital for general health monitoring and so were available for a second tear collection in the following one or two weeks. The premature neonates were resting in an incubator or in their cots, usually in a very warm environment. At the first sample collection, all the premature subjects underwent retinal examination by the investigating ophthalmologist (for retinopathy of prematurity), Mr. Watts. Tear collection was carried out under his supervision and tears were collected before the routine medical exam, while the eye was gently held open by the ophthalmologist.

All the premature infants were seen in the early morning. This was due to Mr. Watts internal arrangement with the hospital clinics.

5.2.2.3 Full-term infants (F1 and F2)

Full-term infants were first investigated in their neonatal period within the first 48 hours of birth (group F1). Some of them agreed to be followed up within the next 4 months (F2).

Full-term infants were usually rested in a cot, although some were held by a parent. If an infant was alert, lids were not kept open manually.

5.2.2.4 Adult subjects (A)

Adult subjects were recruited as a control group. They were members of staff and students of Cardiff University. Local ethical committee approval was obtained for this part of the study, and informed consent was obtained from all subjects prior to participation. Subjects were excluded if they had a systemic or ocular surface disease, took medication known to affect the ocular surface, or if they wore contact lenses, or suffered from an allergy, or were pregnant or had dry eye. Dry eye was defined using a modified McMonnies questionnaire (McMonnies, 1986; McMonnies and Ho, 1987) when one of its described symptoms was found to be present and when fluorescein break-up time of less than 5 seconds was observed. This stringent break-up time limit (Mengher et al., 1985b; Nichols et al., 2002) was chosen for this thesis, to decrease the probability of including subjects with deficient tears.

Similar to previous studies, tear collections were completed before noon to reduce the effect of diurnal variation and also to avoid altered tears by fatigue from prolonged computer use or by laboratory work with various chemicals.

5.2.3 Tear collection

Tear collection and the extraction protocol was used as described in Chapters 4 and 2, respectively. In brief: prior to use, the rods were disinfected by 5 minutes exposure to UV light. Tears were collected with flexible cellulose rods by gently touching the tear

meniscus at the lower lid. Tear collection was rapid and extended only to a maximum of two minutes. To minimise the stress on the infant and to avoid reflex tears, a sample was collected from just one eye, usually the right eye.

5.2.4 Recording and Analysis

5.2.4.1 Biochemical analysis

Tears were first analysed as described in Chapter 3 for their total protein content by the Bradford Coomassie blue test (Bradford 1976) and the distribution of proteins on SDS-PAGE by gel electrophoresis and densitometry of the blue staining. Additionally the tear fern was observed and graded.

5.2.4.2 Tear volume use and measurement

The required tear volume was removed from the sample for total protein concentration assay by the Bradford test, for the distribution of proteins on SDS-PAGE by gel electrophoresis and tear ferning. If any sample was left in the Eppendorf tube, it was measured by pipetting it into another empty tube. Total sample volume was measured by adding used and remaining volume size. Analysis was done on the day of tear collection and any volume remaining was stored at -20°C.

5.2.4.3 Analysis and recording of specific proteins

Major proteins were quantified by densitometry as described in Chapter 3. Their band intensity was compared to the intensity of the known standard. Serum albumin is a less abundant protein and not detectable in adult tears by blue staining (Chapter 4). In

infant tears, it showed a high range of concentrations that was beyond possible linear measurement. The intensity was graded subjectively (Table 5.3).

Table 5.3 Grading system for the serum albumin concentration

Serum albumin band appearance	Grading
no band detected	0
weak	1
medium	2
strong	3

Other identified protein bands such as IgM and lipocalin were graded 0 and 1 according to absence and appearance, respectively, on the gel. The number of bands with intensity above the background was counted after densitometry.

5.2.4.4 Tear ferning

The tear fern was graded according to Rolando et al. (Rolando, 1984) for the entire infant groups and compared to the adult tears.

5.3 RESULTS

5.3.1 RESULTS- Tear volume

Results were checked for normality (SPSS12) and were log-transfered when appropriate to enable parametric tests. Paired testing for the results of infants with repeated tear collection was excluded, based on the insignificant correlation between

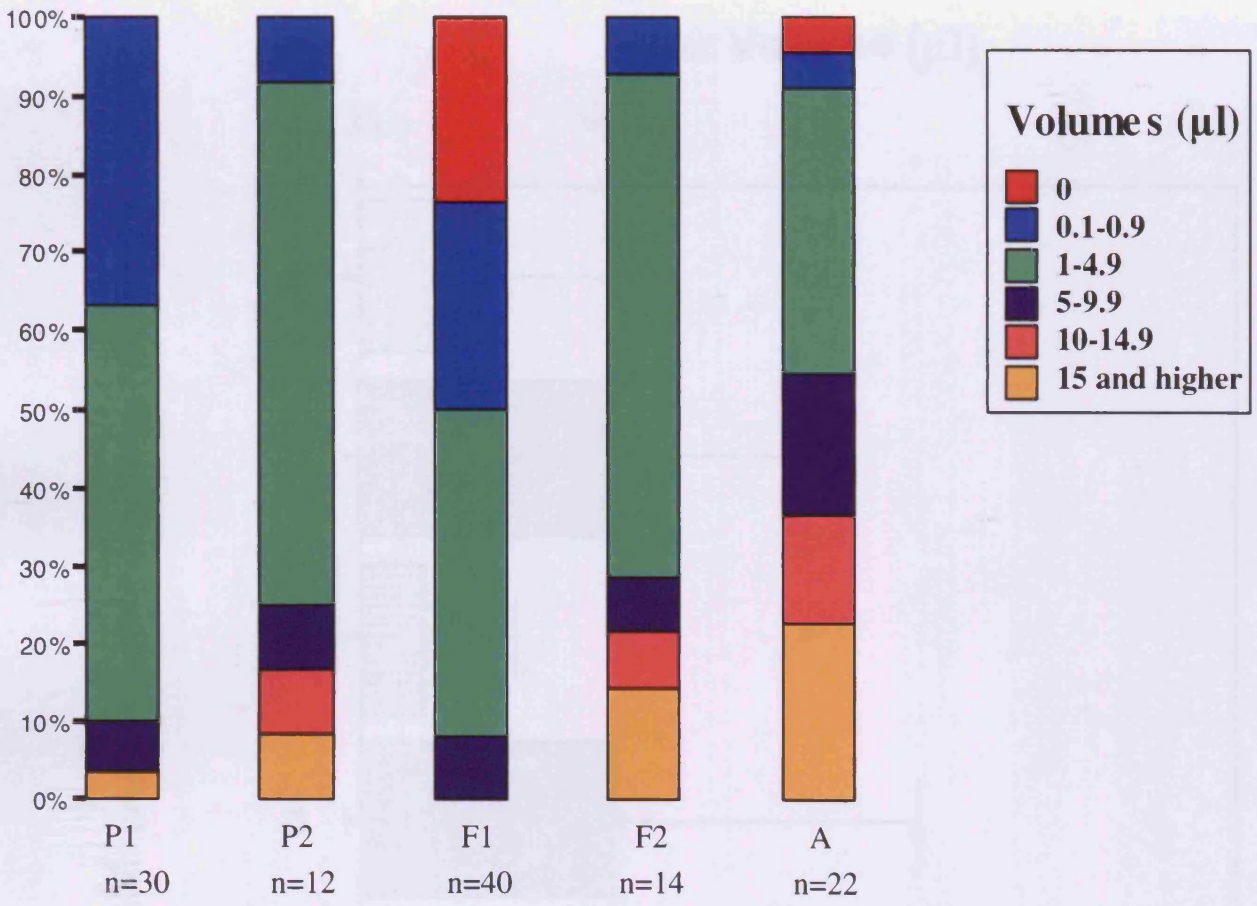
the pairs of results (n=11, r=0.4, p=0.3, correlation testing of paired samples as a subtest of t-test). Paired testing assumes that the two groups are related and therefore a correlation must exist between them. In addition, further analysis of the insufficient paired samples correlation statistics for the premature infant pairs (P1 and P2) showed no statistical power (Excel spreadsheet by Dr. Russell Woods, Schepens Eye Institute, Harvard Medical School). For the unpaired testing of (F1) and (F2), Levene's test of homogeneity of variances showed that variances were equal (p=0.97). This allows comparison of groups with different sample sizes and the means were compared unpaired (power=1).

5.3.1.1 Ability to collect tears for the collection method

The non-normal distribution of the infant tear volume was due to the small volumes collected for some subjects. Different reasons can account for the distribution of volume sizes. Before conducting the analysis, the distribution of no sample or low sample size in each group was observed.

The volume sizes collected between groups were compared. Figure 5.2 shows the variation in volume sizes between the groups. In the premature infant group and the full-term newborn group the distribution is skewed. This is either due to a deficit in the ability of the tear collection or absent tears in these groups[3].

[3] In favour of the first theory, the inability of the collection material, statistical results shown in section 5.3.1.2. exclude volumes with 'zero' µl. However, statistical analysis including 'zero' did not change the significance of the results.



The distribution of collected volume sizes: different volume sizes were grouped in six categories. The percentage of subjects that fall into each category was plotted for all experimental groups.

5.3.1.2 Tear volumes obtained

The mean tear volume (µl±sd) (Figure 5.3) in premature infants (P1) was 2.47±2.95, and in full-term newborn infants (F1) 1.34±1.94. The mean sample volume from the premature group (P2) was 5.01±5, and from the older full-term infants (F2) 5.35±6.37.

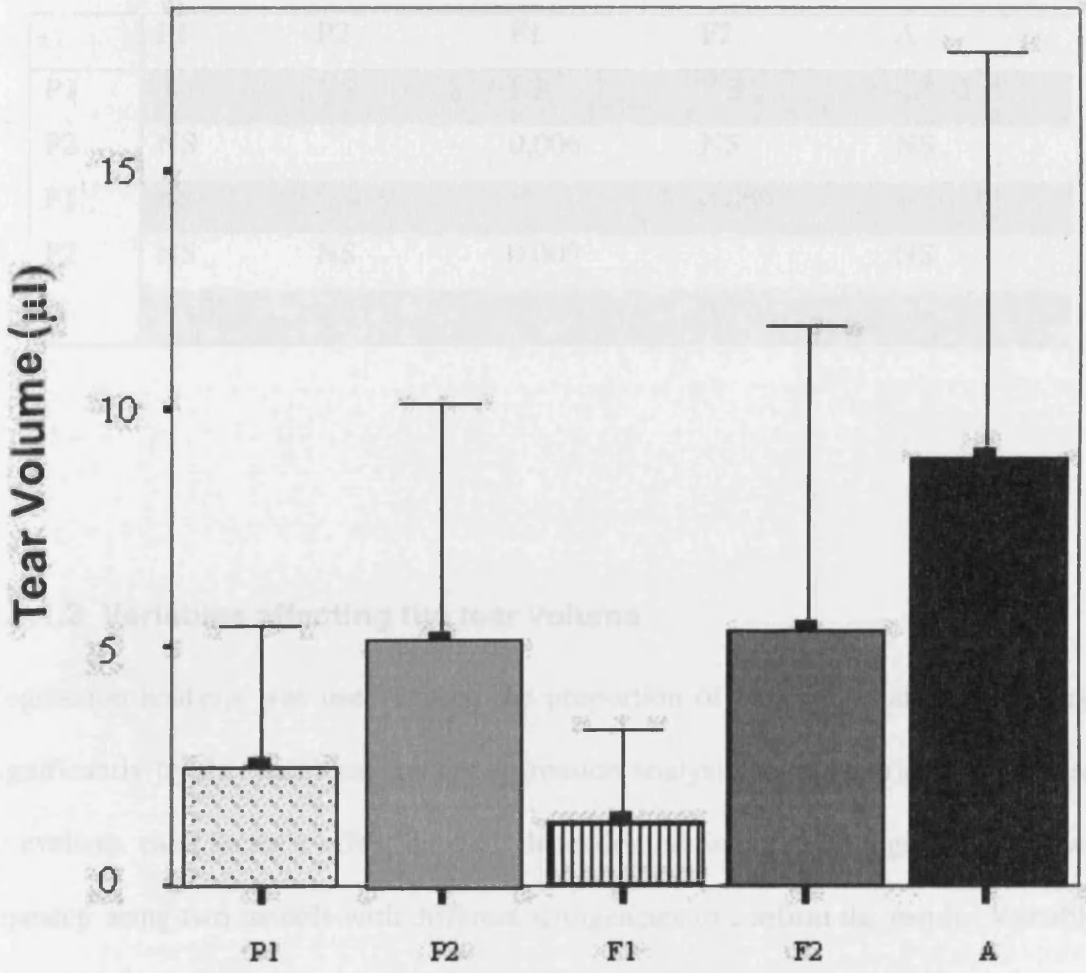


Figure 5.2 Mean±sd tear volume size from different infant groups and adults

In adults (A) the mean (µl±sd) tear volume was 8.98±8.54. Overall a significant difference was found ($p<0.001$, ANOVA one way) based on the differences from post- hoc testing shown in Table 5.4.

Table 5.4 Post hoc (Tukey) test for significant differences in tear volume sizes (NS when p>0.05)

	P1	P2	F1	F2	A
P1		NS	NS	NS	<0.001
P2	NS		0.006	NS	NS
F1	NS	0.006		0.009	<0.001
F2	NS	NS	0.009		NS
A	<0.001	NS	<0.001	NS	

5.3.1.3 Variables affecting the tear volume

Regression analysis was used to find the proportion of variation that was explained significantly by the variables. Linear regression analysis, a parametric test, was used to evaluate each factor's effect on each dependent variable. The regression test was repeated using two models with different stringencies to confirm the result. Variables for maturity, such as GA, PCA, BW and actual weight, other variables, such as gender and alertness were checked for collinearity and excluded if the test was significant. Models were built with remaining variables and statistical results were significant if the model-fit was linear ($p<0.05$). Among the remaining variables only those with a co-efficient of $p<0.05$ contributed significantly to the model. If more than one

variable contributed significantly then the stronger contribution came from the variable with the higher coefficient value β.

Table 5.5 shows the statistical results for the regression analysis of all infant groups. The analysis for the P1 group (n=30) found only a significant model with the variable gender that explains 16% of the variation. Female infants had a higher mean volume size (Figure 5.4). A significant model could also be found for the F2 group (n=14). 72.5% of the variation in the tear volume is explained by the model including the post-conceptional age (PCA). Figure 5.5 shows that the tear volume size increased with increasing PCA.

Table 5.5 Regression analysis for the tear volume size

Infant group	Explained variation (r^2 x 100)	p	Variable contribution
P1 (n=30)	**16%**	**0.03**	**gender (β=0.4 p=0.03)**
P2 (n=12)	49%	0.05	no significant variable
F1 (n=31)	**6%**	**0.8**	**no significant variable**
F2 (n=14)	72.5%	0.003	PCA (β=0.7 p=0.003)

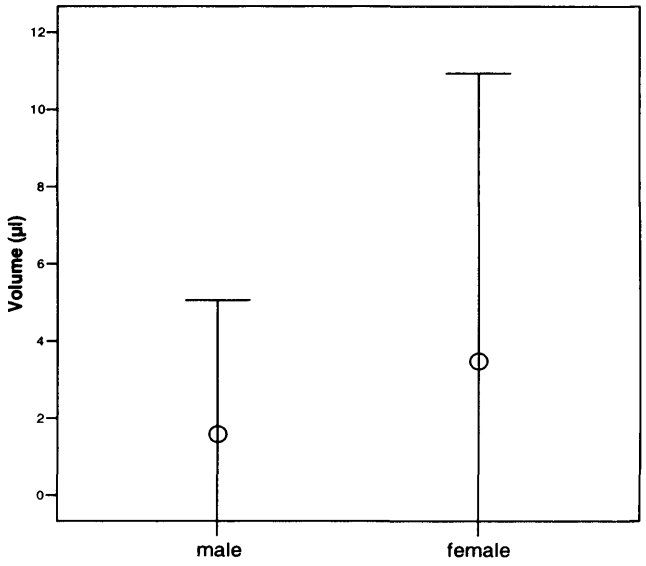


Figure 5.3 The effect of gender on the volume size in the F1 group

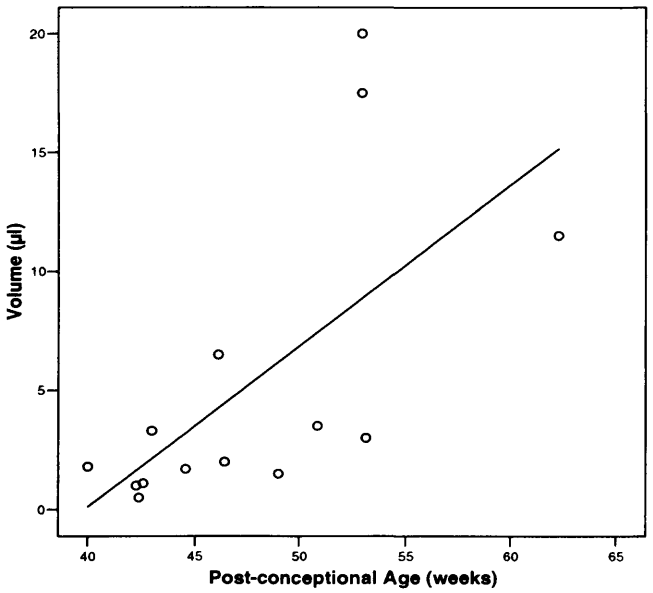


Figure 5.4 Relationship between post-conceptional age and tear volume in full-term infants explained most of the observed variation

5.3.1.4 Volume size differences caused by alertness

During the course of tear collection and extraction it was noticed that some high volume sizes were from these infants. To examine if the alertness state was related to all collected samples the results were regrouped.

Overall 12 infants were crying when tears were collected, 43 infants had their eyes closed and another 36 infants had their eyes open. The test of homogeneity of variances showed equal variances (p=0.5) and allowed parametric testing of the log-transformed results. The tear volume differed according to the state of the infants (p=0.04, ANOVA one way). The main difference was found between volumes sampled from crying and closed eye tear volume size (p=0.046, Tukey). The tear volume of crying infants was increased (Figure 5.6).

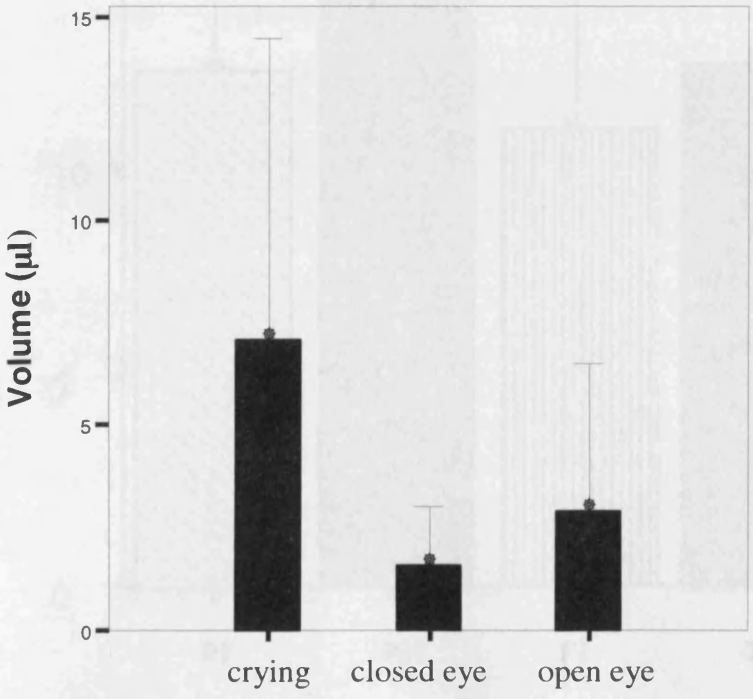


Figure 5.5 Mean±sd tear volume compared by infants' state of alertness

5.3.2 RESULTS- Protein concentrations

All results were examined for their normality and appropriate statistical tests were applied (SPSS 12). Mean total tear protein concentrations (µg/µl ±sd) are shown in Figure 5.7 for premature infants P1 (n=15), the full-term newborn infants F1 (n=8), the premature group F2 (n=7), the full-term infants F2 (n=11) and the adult group (n=9). Not all samples had enough volume size for the protein analysis. This explains the different numbers (n) for each group. No significant difference was found in protein concentrations between any of the groups (p=0.8, ANOVA one way).

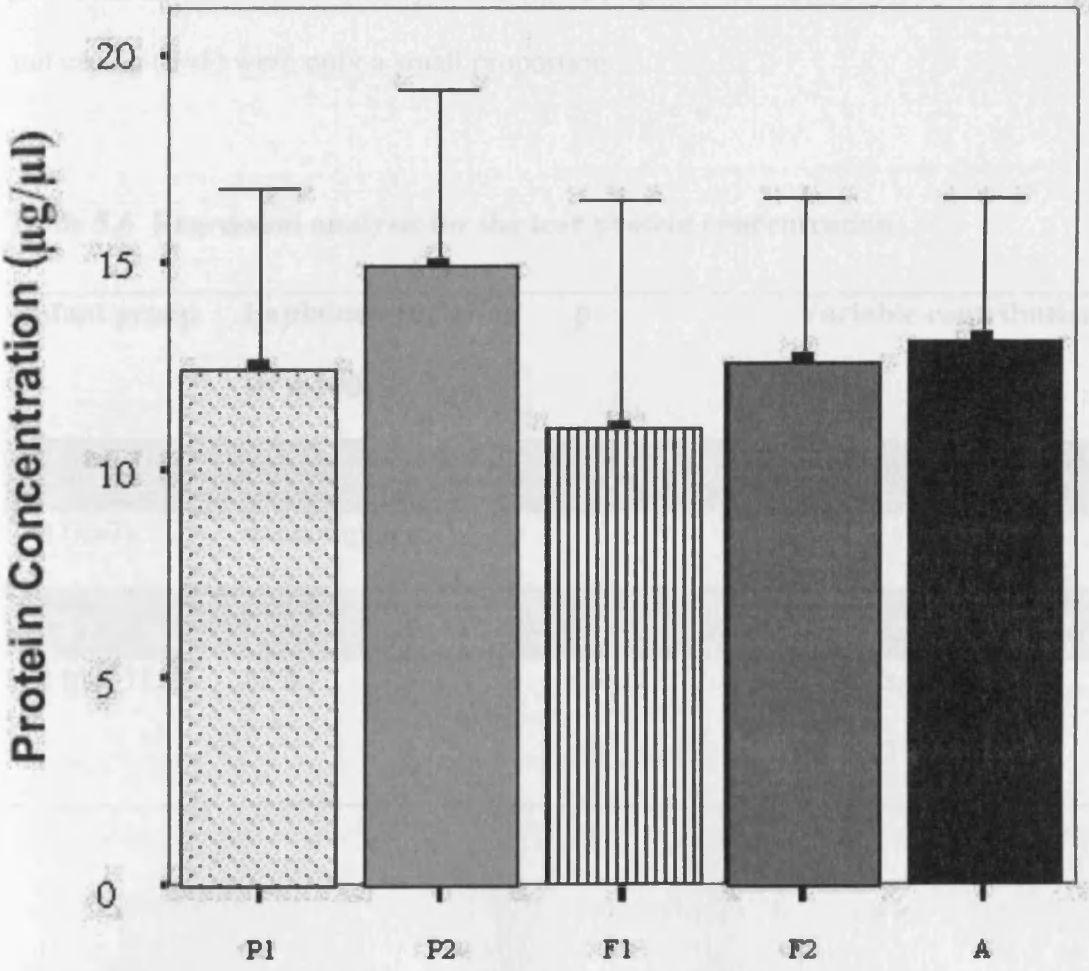


Figure 5.6 Protein concentrations (mean±sd) in the experimental groups

5.3.2.1 Variables affecting the protein concentration

The relationship between the variables for maturity, gender, sample volume size, alertness and the protein concentration was examined by linear regression analysis. Statistical results are shown in Table 5.6.

The number of results in group P2 and F1 was too small to conduct statistical analysis. No significant model was found for P1. In the older full-term infants F2 a significant model with actual weight, gender and alertness explained 82% of the variation. Alertness had the only significant contribution to this model with decreased protein concentration in open eye tears (n=7) (Figure 5.8). However, closed eye (n=3) and crying (n=1) were only a small proportion.

Table 5.6 Regression analysis for the tear protein concentration

Infant group	Explained variation (r^2 x 100)	p	Variable contribution
P1 (n=15)	23%	0.7	no significant variable
P2 (n=7)	not computed		
F1 (n=8)	not computed		
F2 (n=11)	82%	0.002	alertness (β=-0.73, p=0.02)

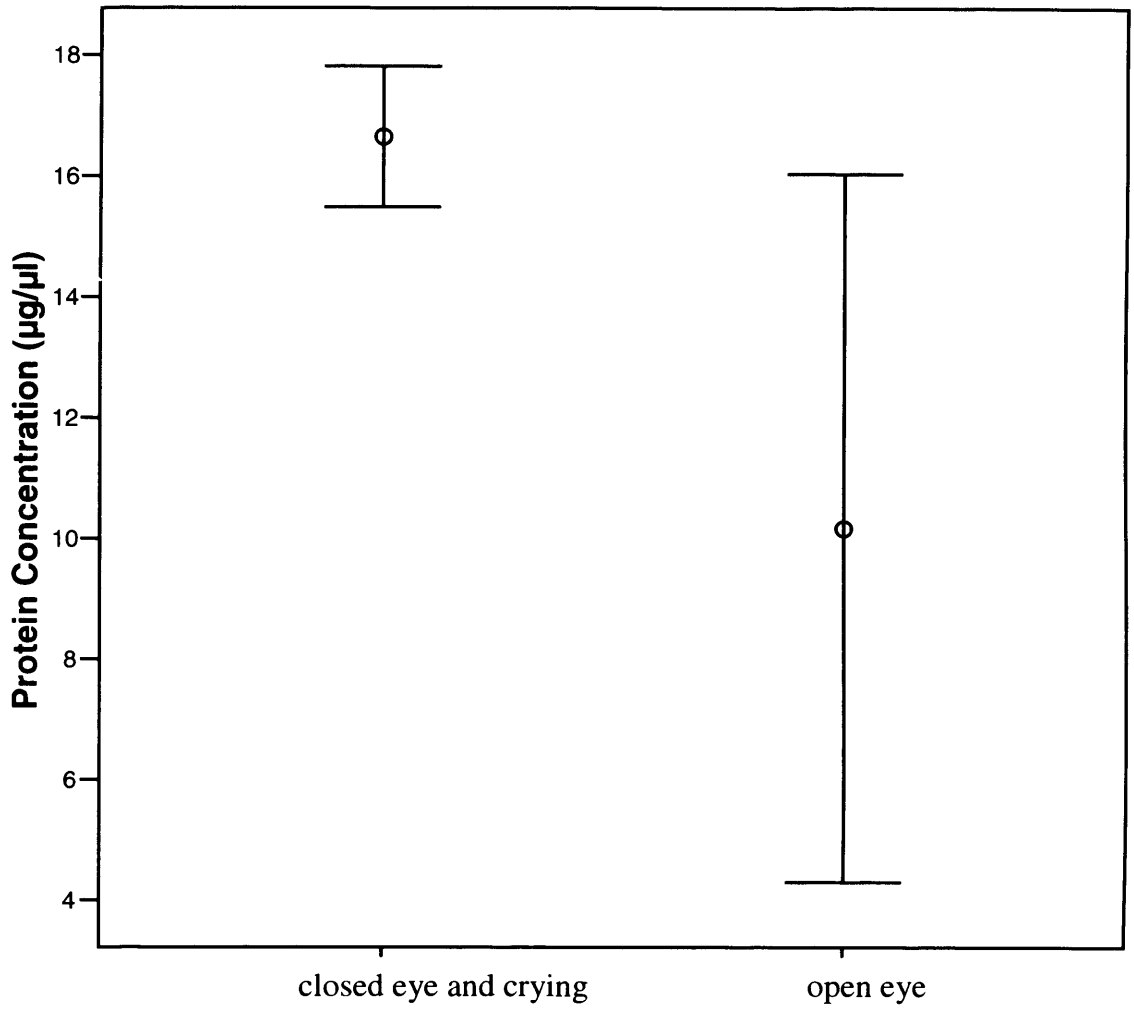


Figure 5.7 Relationship of alertness and protein concentration

In adults, it is established that closed and open tears are different in their protein content (Sack et al., 2000). In the next section the effect of open eye, closed eye and crying were further investigated to test if there was an overall effect on the protein concentration regardless of the maturity of the infants. To examine if the variable "alertness" made a difference on the protein concentration, the separation into infant groups was abandoned and results were regrouped by crying, open and closed eye tears.

5.3.2.2 Protein concentration differences caused by alertness

Overall, protein concentration results were obtained for 5 crying infants, 17 closed eye infants, and 19 open eye infants from available tear volume size for analysis. A significant difference in protein concentration was found (p=0.014, ANOVA one-way) with the significant difference between closed eye and open eye tear concentration (p=0.017, Tukey). The tear protein concentration in infants with closed eyes was increased (Figure 5.9).

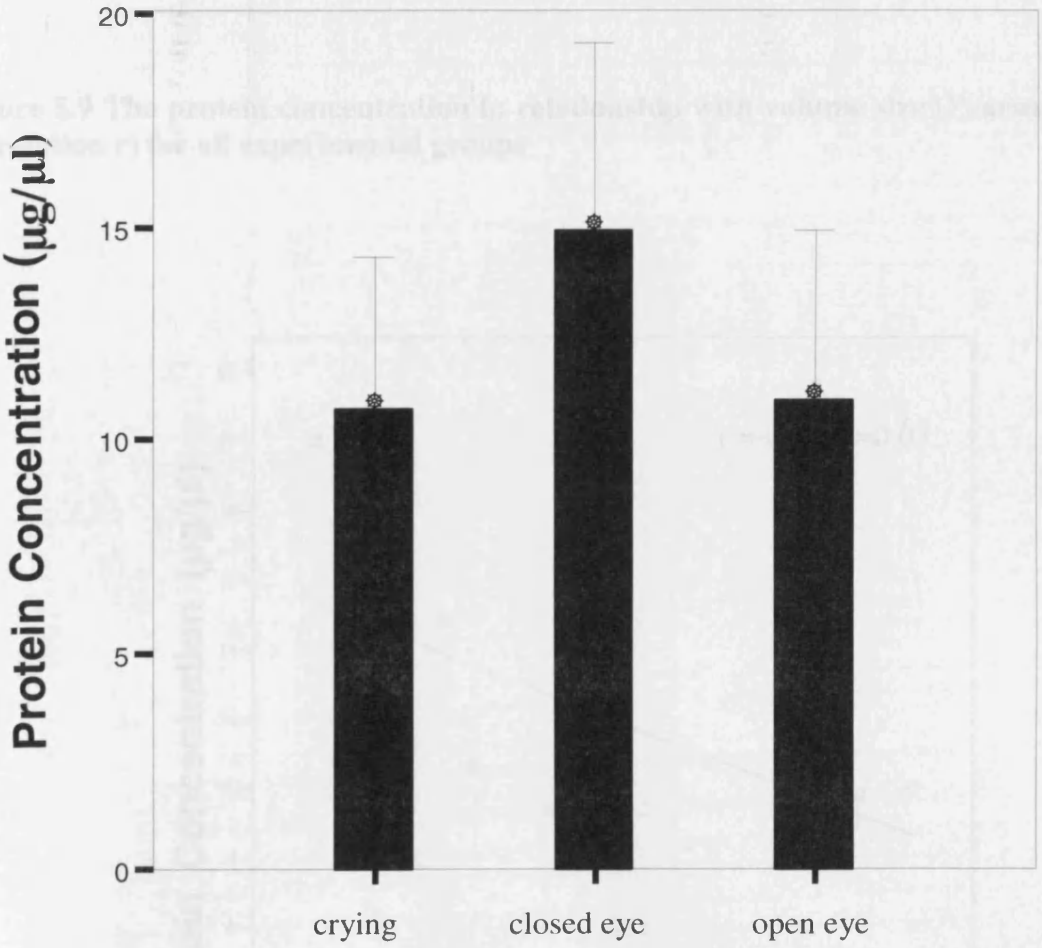


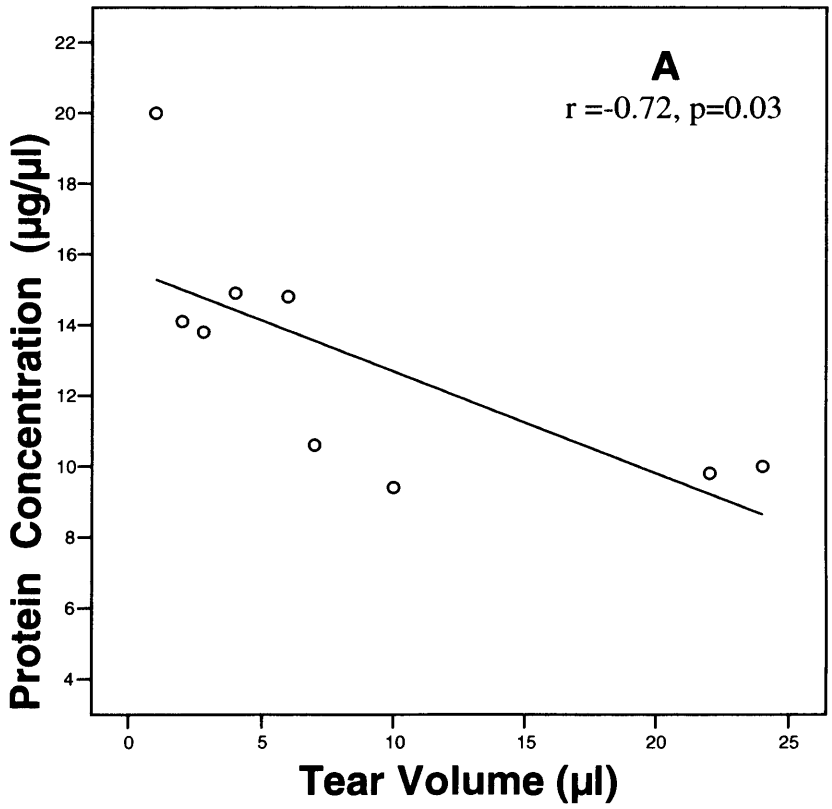
Figure 5.8 Mean±sd protein concentrations compared by infants' state of alertness

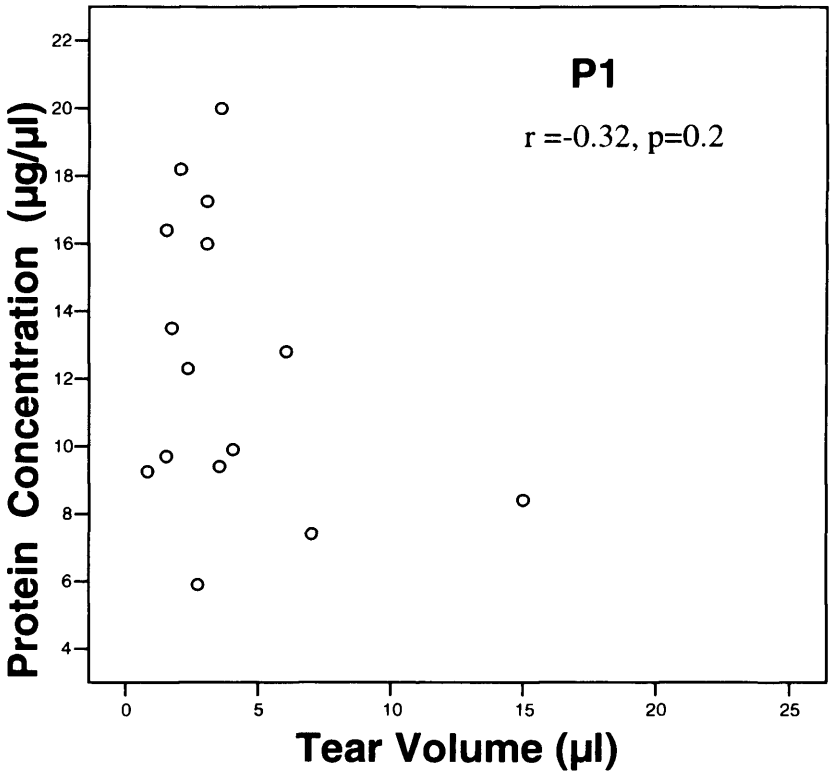
5.3.3 RESULTS- Protein concentration changes with volume size

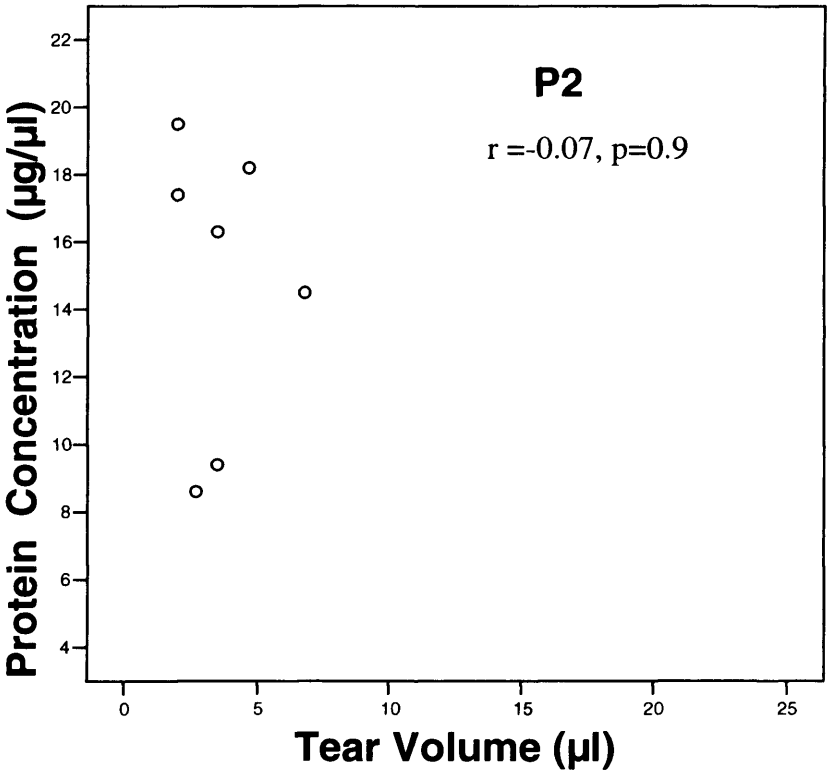
In Chapter 4, Experiment 5, it was shown that larger tear volumes contain less protein. This relationship was found between the adult tear volume and protein concentration (Figure 5.10 for group A). However, no linear relationship existed for the infant groups' tear volume and protein concentrations (Figure 5.10 for P1, P2, F1 and F2).

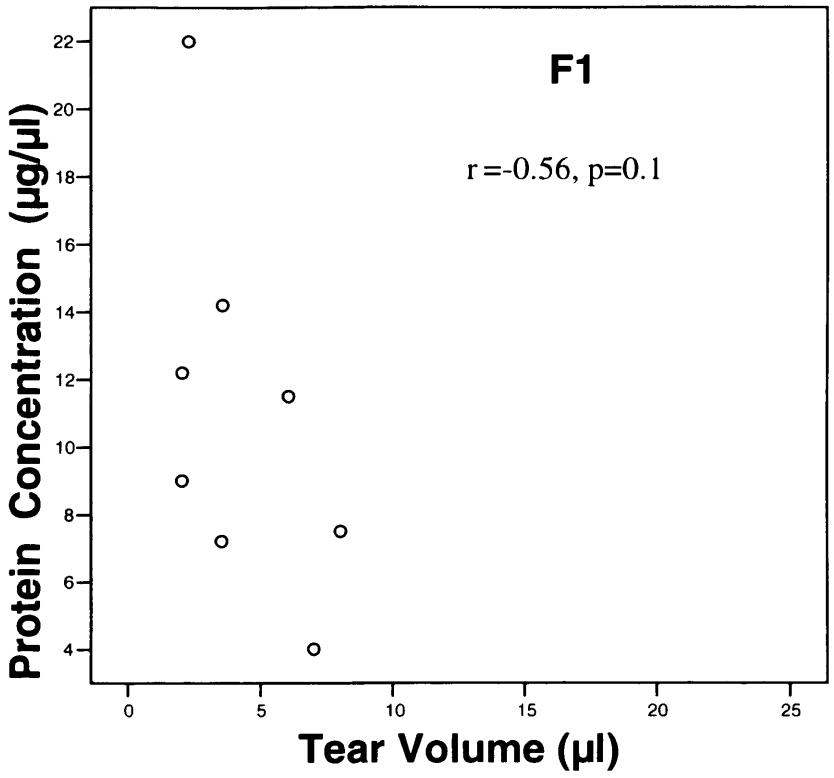
Other curve estimations for non-linear relationships did not help to improve the curve fit. Excluding crying infants also did not allow for finding a relationship.

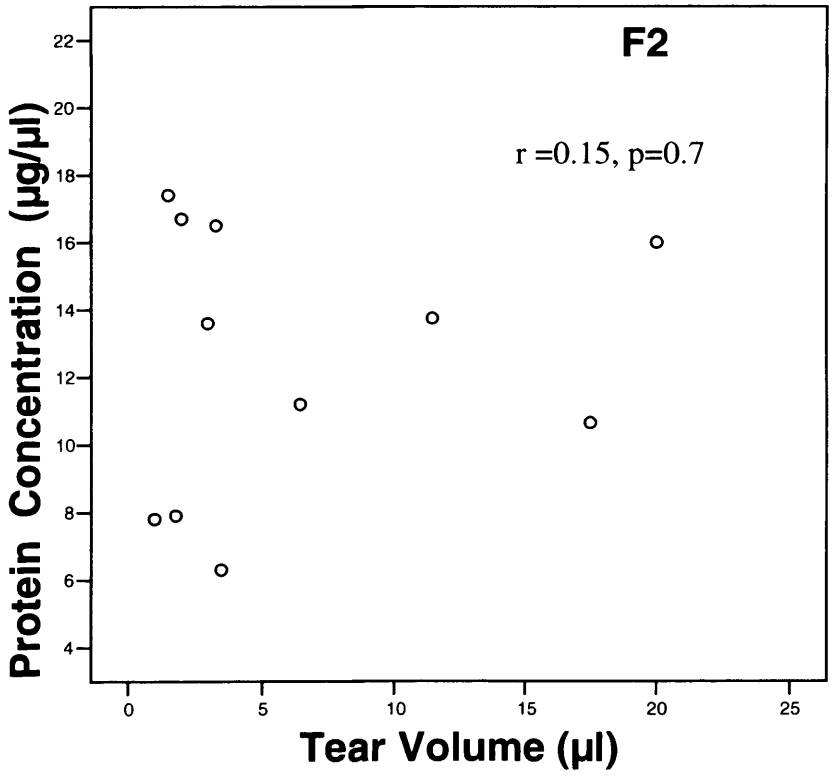
Figure 5.9 The protein concentration in relationship with volume size (Pearson's correlation r) for all experimental groups




P1
r =-0.32, p=0.2
Protein Concentration (µg/µl)
Tear Volume (µl)
22
20
18
16
14
12
10
8
6
4
0
5
10
15
20
25


P2
r =-0.07, p=0.9
Protein Concentration (µg/µl)
Tear Volume (µl)
22
20
18
16
14
12
10
8
6
4
0
5
10
15
20
25


F1
r =-0.56, p=0.1
Protein Concentration (µg/µl)
Tear Volume (µl)
22
20
18
16
14
12
10
8
6
4
0
5
10
15
20
25


F2
r =0.15, p=0.7
Protein Concentration (µg/µl)
Tear Volume (µl)
22
20
18
16
14
12
10
8
6
4
0
5
10
15
20
25

5.3.4 RESULTS- Analysis of major proteins

In this section the protein concentration of IgA, lactoferrin and lysozyme are presented for each subgroup and compared with each other. The results examined the effect of known parameters on the concentration. SDS-PAGE was performed when sufficient volume size was available. Therefore results were obtained for

- n=23 in group P1
- n=11 in group P2
- n=22 in group F1
- n=13 in group F2
- n=19 in group A

5.3.4.1 IgA concentration

The distribution of IgA concentrations was tested for normality and was found to be normal after log-transformation. Mean IgA concentrations were observed (Figure 5.11) and found to differ significantly between the experimental groups (p=0.001, ANOVA one-way) based on the differences from post-hoc testing shown in Table 5.7).

Table 5.7 Post-hoc (Tukey) test for significant differences in IgA concentrations (NS when p>0.05)

	P1	P2	F1	F2	A
P1		NS	0.002	NS	NS
P2	NS		0.04	NS	NS
F1	0.002	0.04		NS	<0.001
F2	NS	NS	NS		NS
A	NS	NS	<0.001	NS	

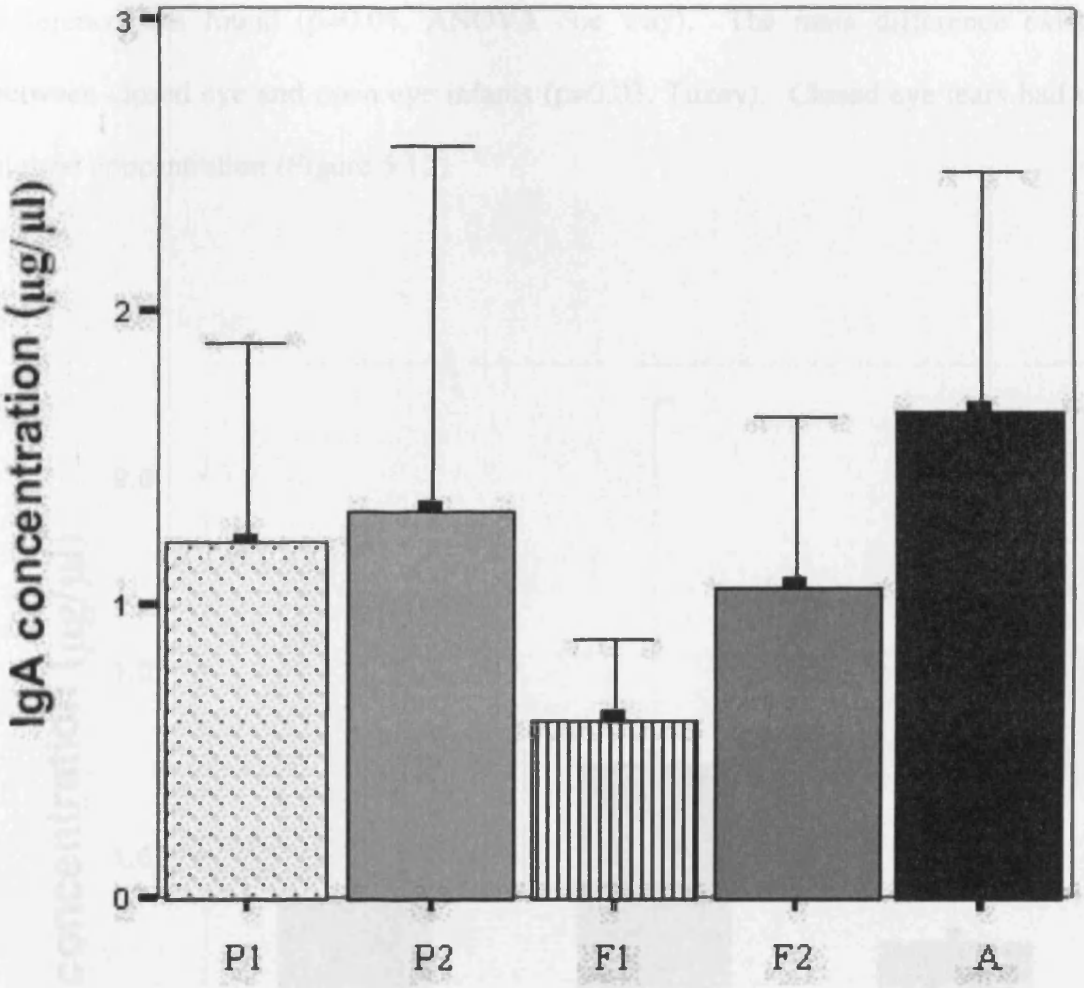


Figure 5.10 Mean±sd of infants' and adults' IgA concentration

5.3.4.2 Variables affecting the IgA concentration

To investigate the effect of alertness and to determine which state made a difference to the IgA concentration, the log-transformed means were compared. From the twelve infants that were crying, n=8 samples were large enough for SDS-PAGE. From 43 infants with closed eyes, n=32 samples were analysed and from 36 open eye

infants, n=29 samples were analysed for their major protein content. The test of homogeneity of variances showed equal variances (p=0.3) and allowed parametric testing of the log-transformed IgA results. Amongst the infants a significant difference was found (p=0.04, ANOVA one way). The main difference existed between closed eye and open eye infants (p=0.03, Tukey). Closed eye tears had the highest concentration (Figure 5.12).

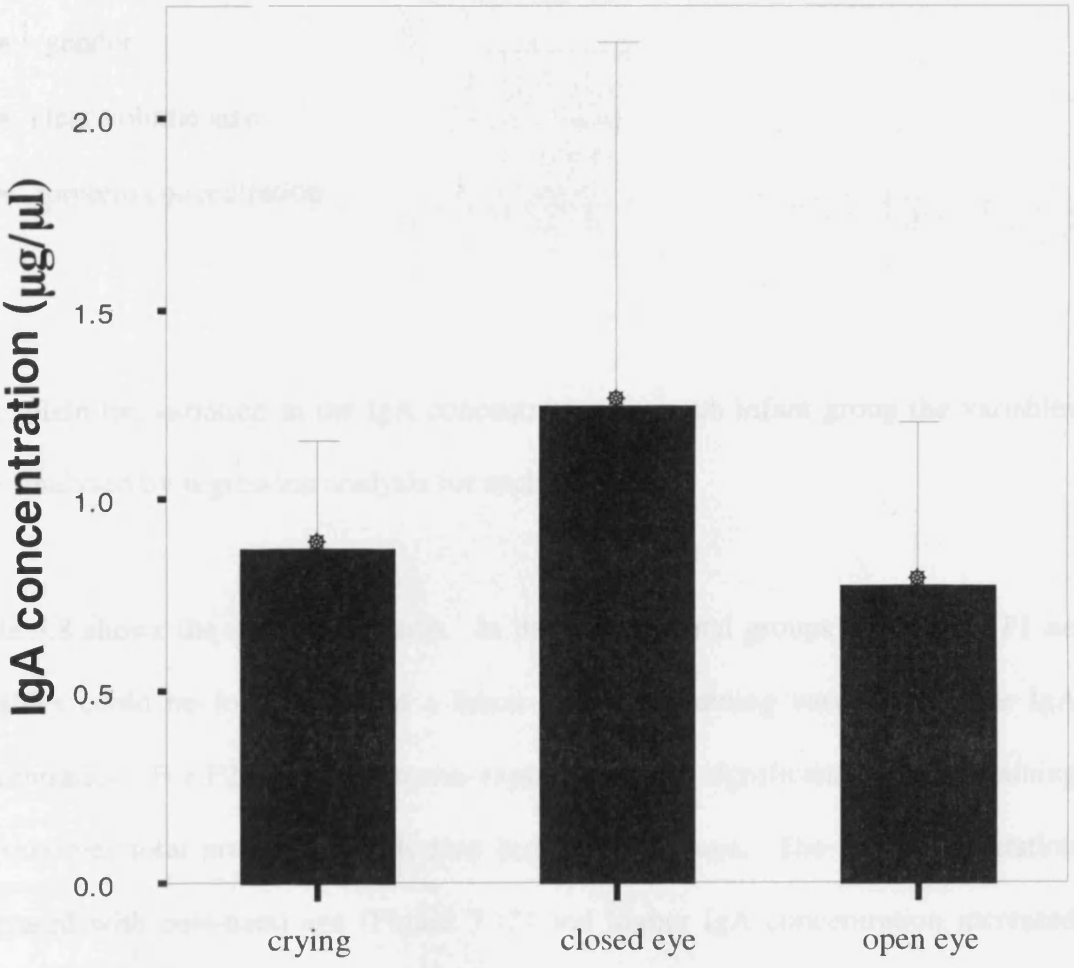


Figure 5.11 Effect of alertness on the IgA concentration (mean±sd)

The log-transformed IgA concentration results for all infants (n=69) were subjected to regression analysis to find a variable that contributed most to its variation. The variables were

- GA
- PCA
- BW
- actual weight
- gender
- tear volume size
- protein concentration

To explain the variation in the IgA concentration for each infant group the variables were analysed by regression analysis for each group.

Table 5.8 shows the statistical results. In the experimental groups P1, P2 and F1 no variables could be found to build a linear model explaining variations in the IgA concentration. For F2 the variation was explained with a significant model containing the variables total protein concentration and post-natal age. The IgA concentration decreased with post-natal age (Figure 5.13) and higher IgA concentration increased the total protein concentration (Figure 5.14).

Table 5.8 Regression analysis for the IgA concentration

Infant group	Explained variation (r^2 x 100)	p	Variable contribution
P1 (n=23)	**37%**	**0.7**	**no significant variable**
P2 (n=11)	17%	0.8	no significant variable
F1 (n=22)	**69%**	**0.4**	**no significant variable**
F2 (n=13)	87%	<0.001	post-natal age (β=-0.81, p<0.001) protein concentration (β=0.67, p=0.001)
A (n=19)	**24%**	**0.1**	**no significant variable**

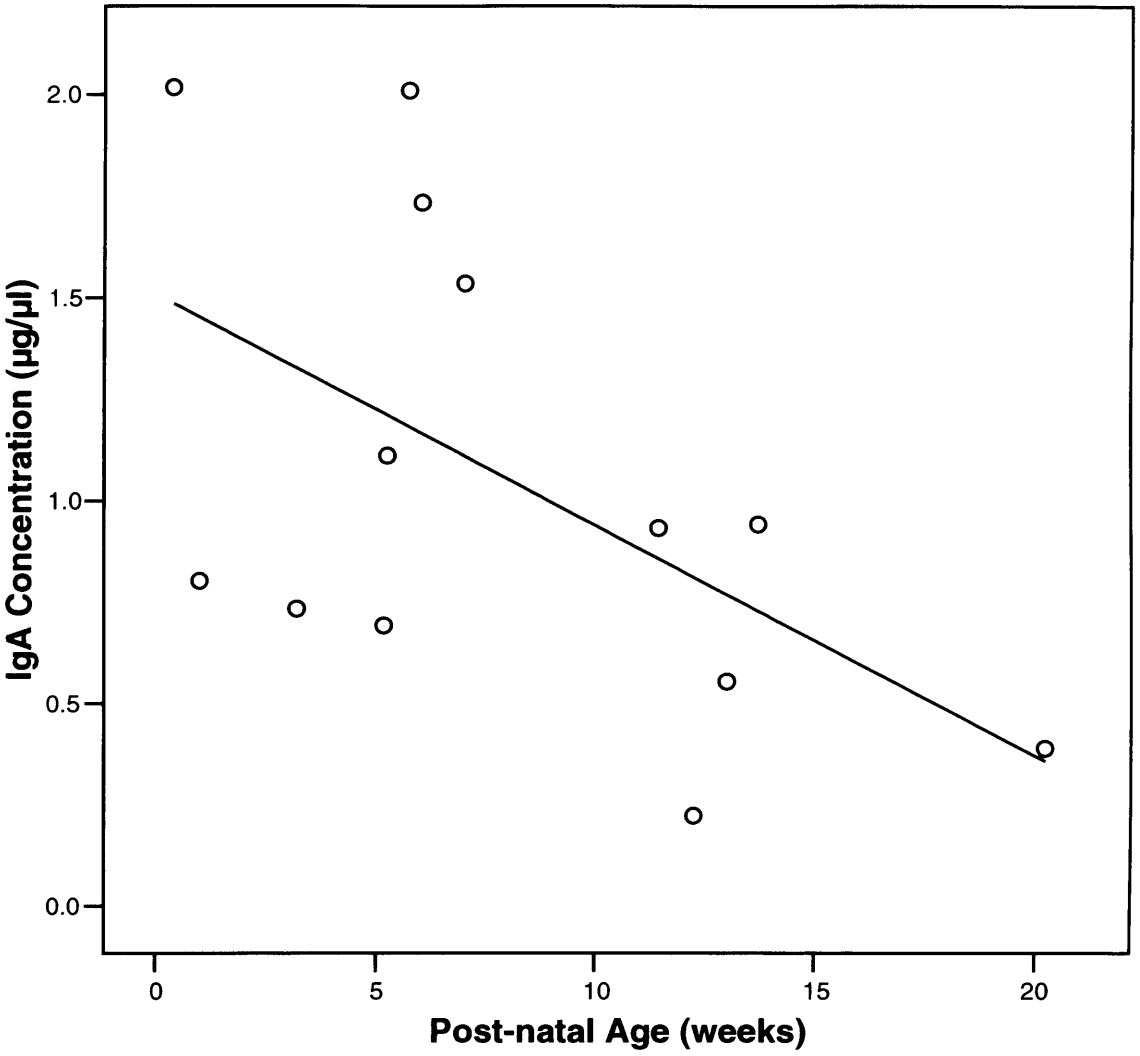


Figure 5.12 Effect of age on the IgA concentration of the F2 group

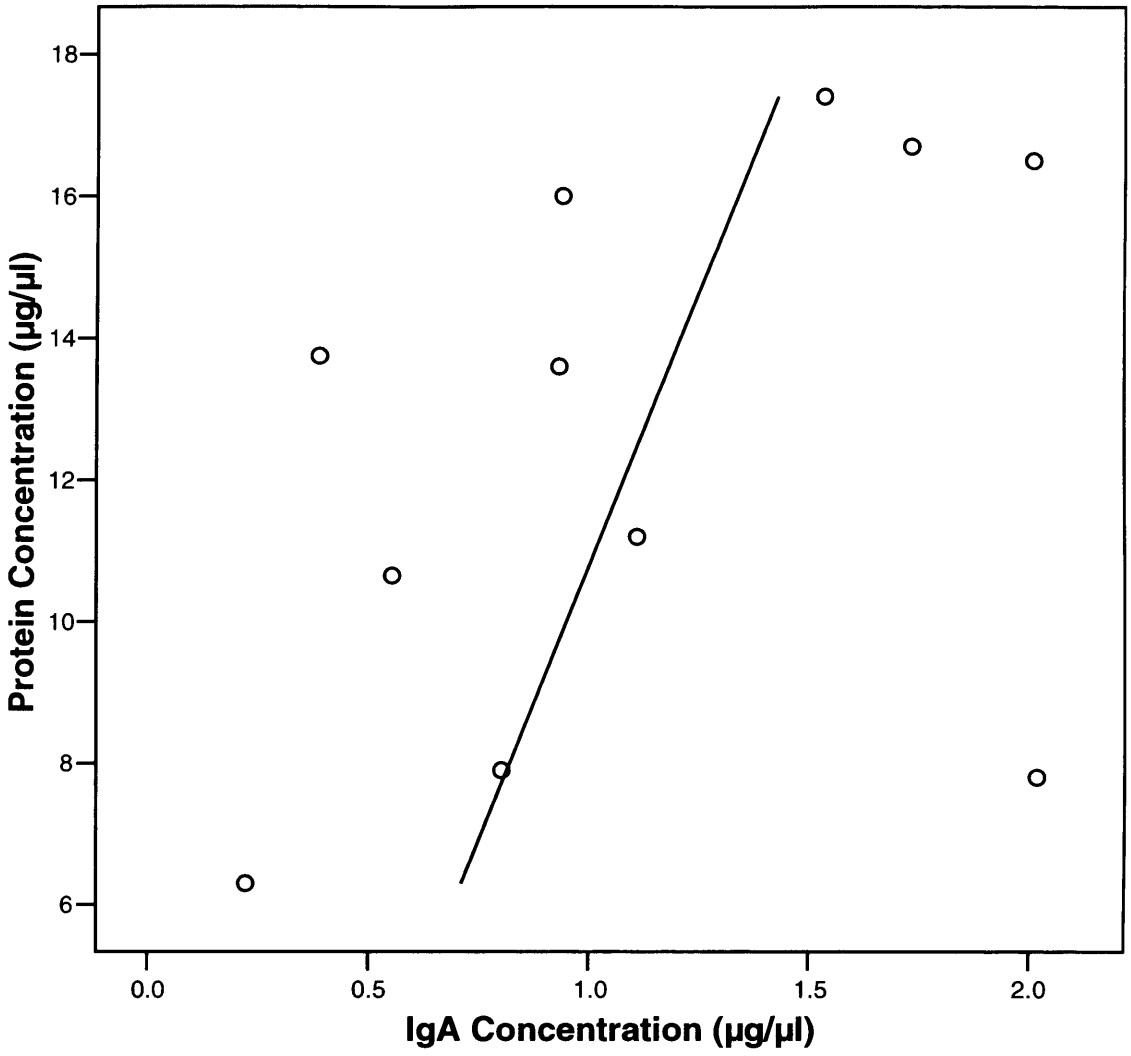


Figure 5.13 Relationship between the IgA and the total protein concentration of the F2 group

5.3.4.3 Lactoferrin concentration

Prior to analysis the results were tested for normality by appropriate methods. The lactoferrin concentration did not differ between the experimental groups (p=0.05, ANOVA one way) (Figure 5.15).

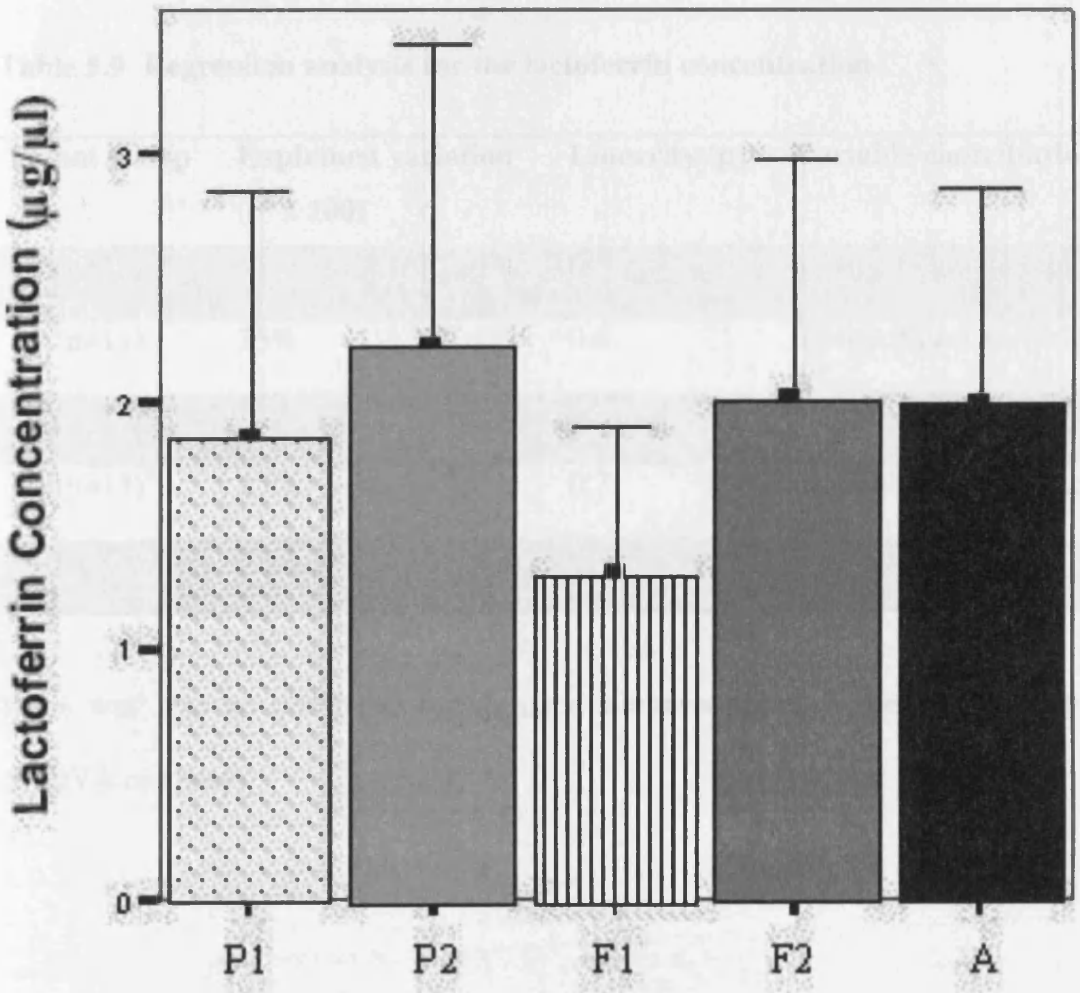


Figure 5.14 Mean±sd of infants' and adults' lactoferrin concentration

5.3.4.4 Variables affecting the lactoferrin concentration

As for the IgA concentration, variables for maturity, gender, volume size and protein concentration were investigated for their effect on the lactoferrin variation. No regression model with any combination of these variables could explain variations in lactoferrin concentration for each experimental group (Table 5.9).

Table 5.9 Regression analysis for the lactoferrin concentration

Infant group	Explained variation (r^2 x 100)	Linearity (p)	Variable contribution
P1 (n=23)	48%	0.3	no significant variable
P2 (n=11)	35%	0.6	no significant variable
F1 (n=22)	81%	0.4	no significant variable
F2 (n=13)	25%	0.7	no significant variable
A (n=19)	27%	0.08	no significant variable

There was also no difference between the alertness states of the infants (p=0.26, ANOVA one-way).

5.3.4.5 Lysozyme concentration

Prior to analysis the results were tested for normality by appropriate methods. No difference was found between the lysozyme concentration of all groups (p=0.131, ANOVA one-way) (Figure 15.6).

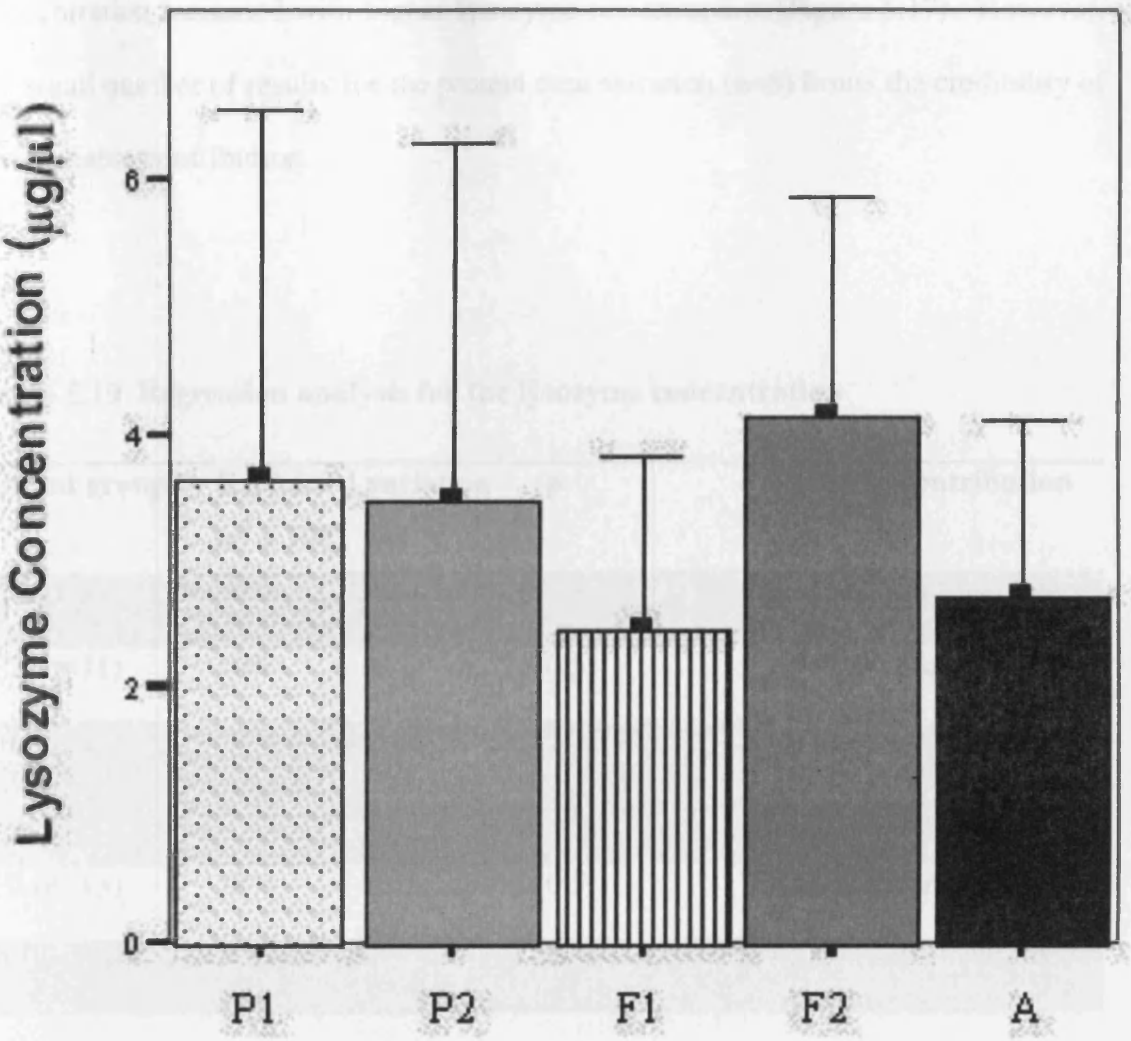


Figure 5.15 Mean±sd of infants' and adults' lysozyme concentration

5.3.4.6 Variables affecting the lysozyme concentration

Regrouping of the results to test effects of alertness did not show any overall difference (p=0.2, ANOVA one-way). Regression analysis, similar to IgA and lactoferrin, was used with variables for maturity, gender, volume size and protein concentration. No regression model with any combination of these variables could explain variations in lysozyme concentration for adults and groups P1, P2 and F2 (Table 5.10). In group F1 a significant model was found. The total protein

concentration increased with higher lysozyme concentration (Figure 5.17). However, the small number of results for the protein concentration (n=8) limits the credibility of this variable contribution.

Table 5.10 Regression analysis for the lysozyme concentration

Infant group	Explained variation (r^2 x 100)	p	Variable contribution
P1 (n=23)	**26%**	**0.3**	**no significant variable**
P2 (n=11)	26%	0.5	no significant variable
F1 (n=22)	**55%**	**0.04**	**protein concentration (β=0.7, p=0.04)**
F2 (n=13)	28%	0.5	no significant variable
A (n=19)	**32%**	**0.05**	**no significant variable**

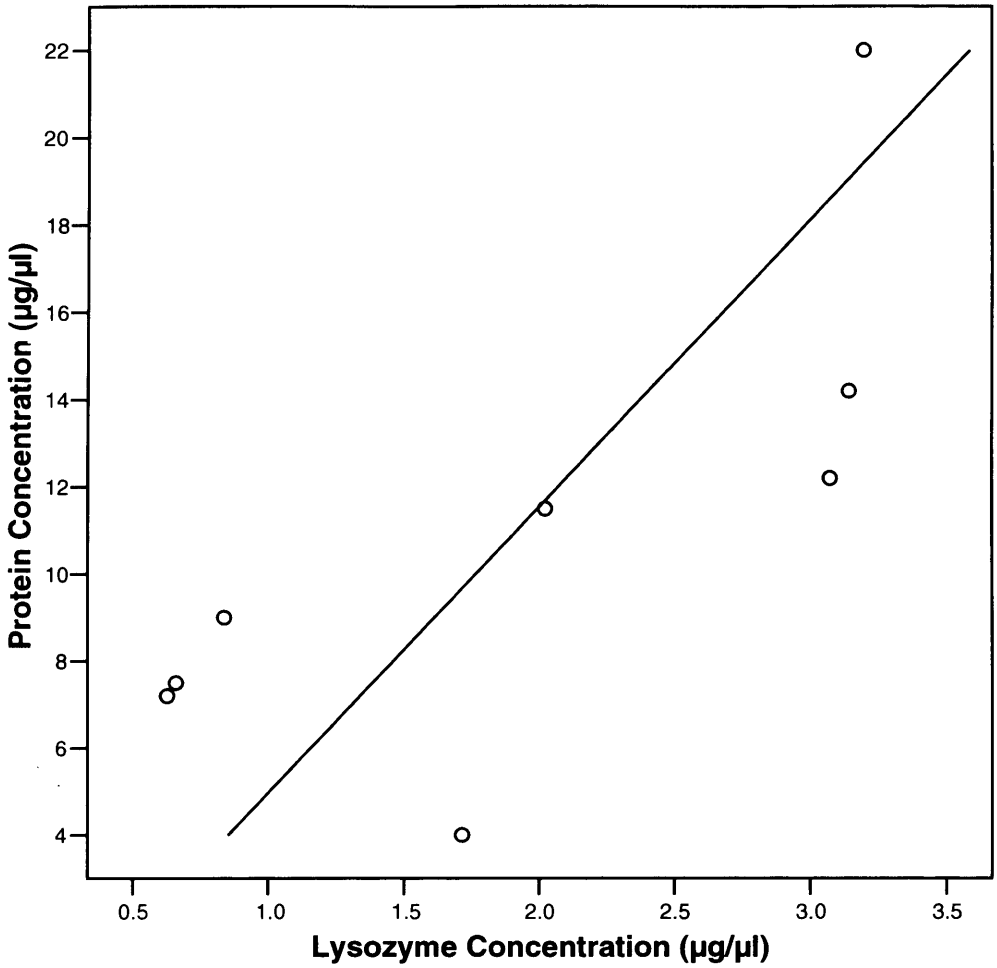


Figure 5.16 Effect of the lysozyme concentration on the total protein concentration

5.3.5 RESULTS- Observation of the protein profile

After measuring the three main proteins, the protein profiles were observed for the presence of other known proteins. These were identified as lipocalin, serum albumin, and IgM. The number of bands on the gel was also examined. These factors are evaluated and compared between groups and for variation caused by the known parameters.

5.3.5.1 Lipocalin

All samples from the adult group contained enough lipocalin to present a band on the SDS-PAGE. It was noticed that in some infant tear samples this band was not present. The presence of the lipocalin bands for each experimental group is shown in Table 5.11. The non-parametric equivalent to the one-way ANOVA was used to test for differences between the infant groups. No significant difference was found in the frequency of present lipocalin band between all infant groups (p=0.145, Kruskal-Wallis test).

Table 5.11 Frequency of lipocalin presence in different infant groups

Groups	P1 n=23	P2 n=11	F1 N=22	F2 n=13	A n=19
present	30%	70%	36%	54%	100%
not present	70%	30%	64%	46%	0%

5.3.5.2 Variables affecting the lipocalin presence

To examine whether the variables maturity, gender, volume size, and protein concentration can predict the lipocalin presence, logistic regression (binary) for dichotomous (when results are 1=present and 0=not present) dependents was used. Two stepwise methods for entering the variables were chosen, to duplicate the

analysis. This was intended to confirm the results from the two methods. These methods rejected or included variables that were significant for the model. When variables were included, a model was built to explain the proportion of variation in the results by the size of Nagelkerke's pseudo r^2. This fit was examined with the Hosmer and Lemeshow test. The model fits well when the p value is not significant (>0.05).

For group P1 a model was fitted (r^2= 28, Nagelkerke; p=0.8, Hosmer and Lemeshow test) that predicted 28% of present cases and 100% of absent cases correctly. This model included volume (p=0.03). Figure 5.18 shows that with smaller volumes the probability of lipocalin absence increases, whereas its presence can be found in a wider range of tear volume sizes.

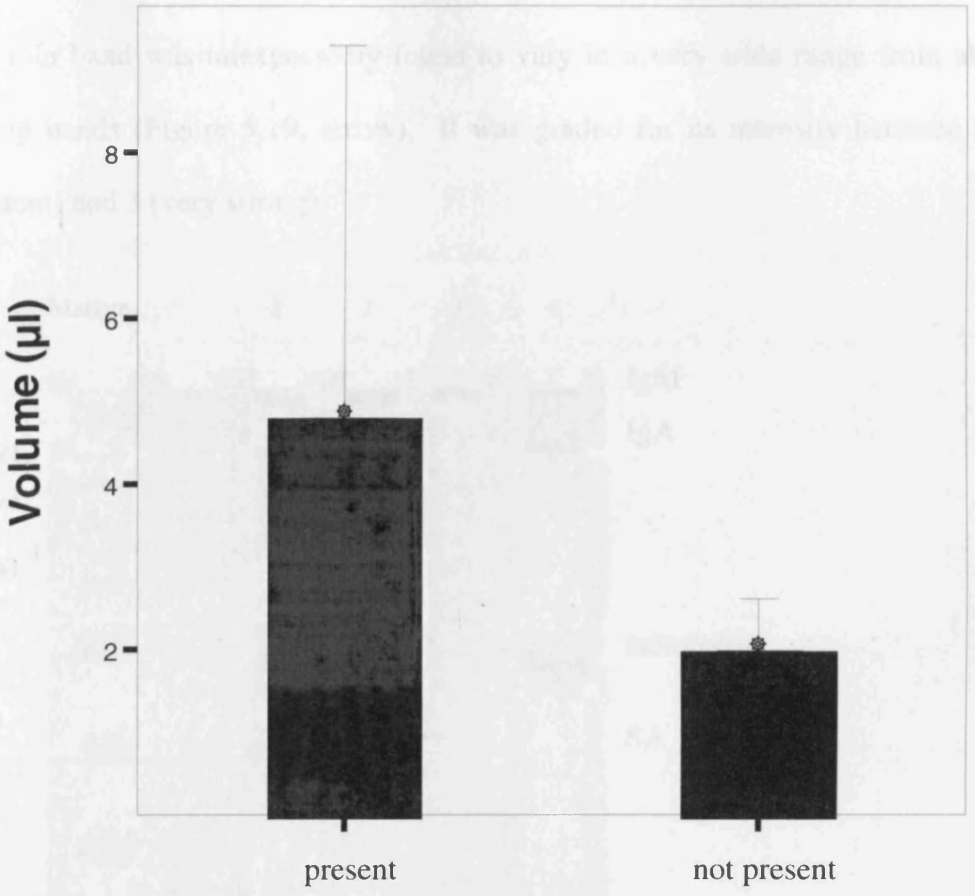
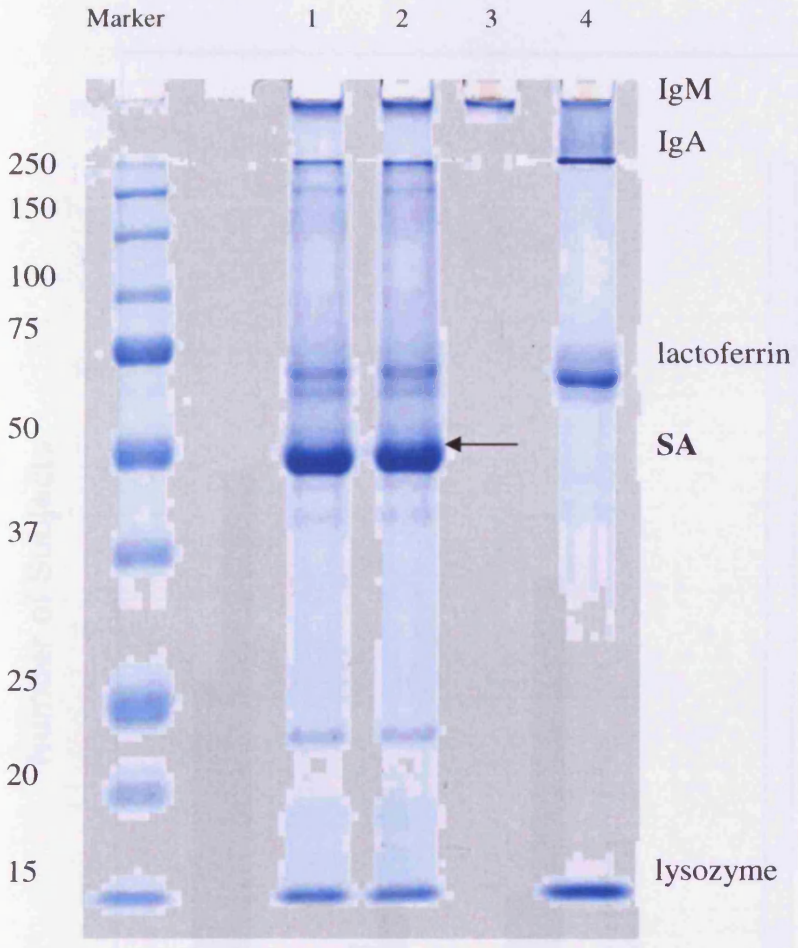


Figure 5.17 Tear volume size of the lipocalin presence on the SDS-PAGE: bars represent mean±sd

Logistic regression for the groups P2, F1 and F2 was not able to include any of the variables to build a model (for each variable $p>0.05$, Wald statistics). Regrouping of the infants to find an overall effect of the variables for alertness did not show any significant difference ($p=0.5$, Kruskal-Wallis test).

5.3.5.3 Serum albumin

In adult tears serum albumin is a low abundance protein. In infant tears the serum albumin band was unexpectedly found to vary in a very wide range from absent to strong bands (Figure 5.19, arrow). It was graded for its intensity between 0 (non-existent) and 3 (very strong).



Lanes:	
1 and 2:	tears from a premature infant
3:	IgM standard
4:	IgM, IgA, lactoferrin, lysozyme standards

Figure 5.18 Tear protein profile of premature infant tears (arrow indicates SA band)

Figure 5.20 demonstrates the SA distribution of all infants and adults in four grades from 0-3. To test for differences between the infant groups the non-parametric equivalent of the one-way ANOVA was used. A significant difference was found between the SA concentration of premature infants, newborn full-term infants, full-term infants ($p<0.001$, Kruskal-Wallis test).

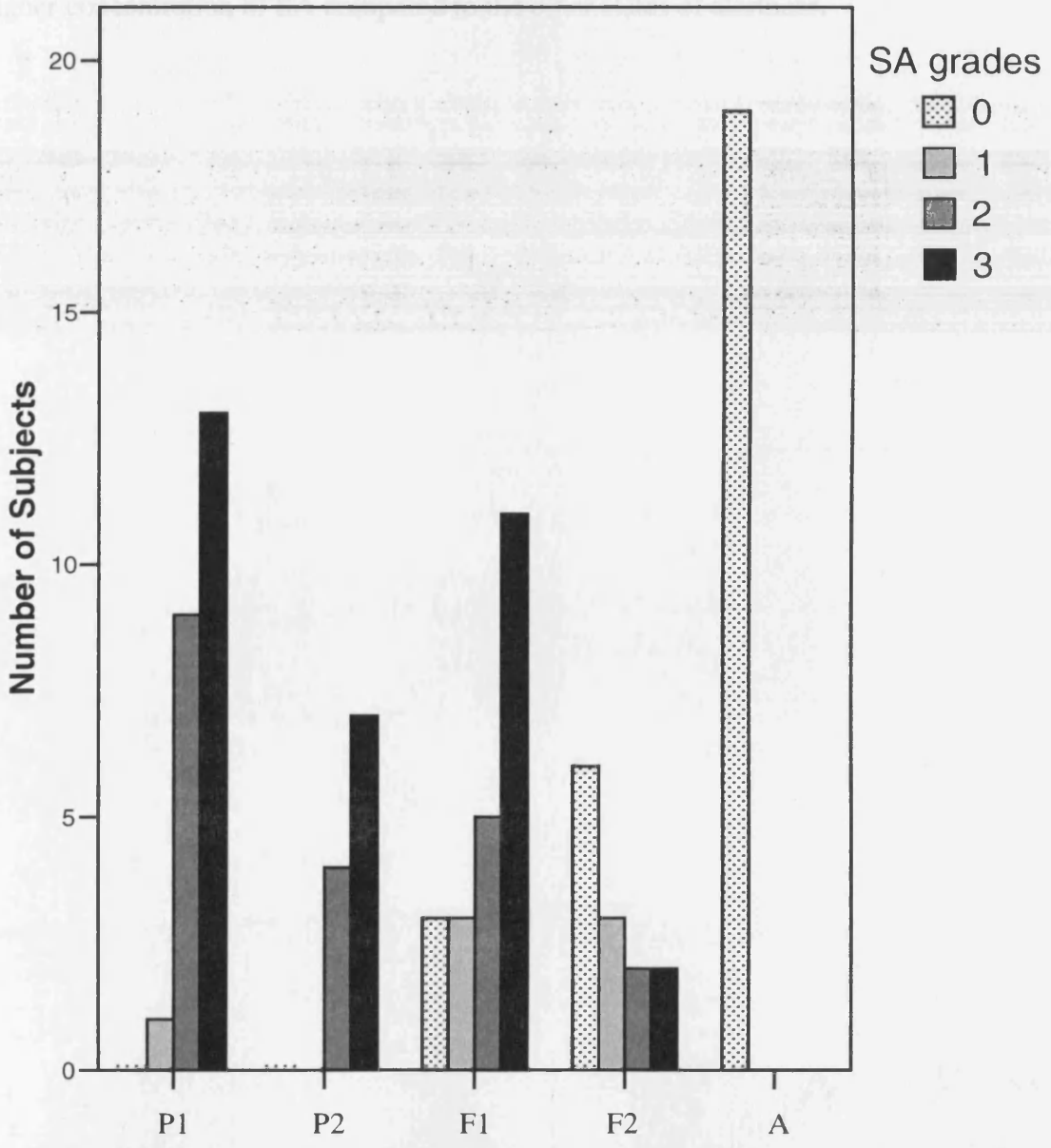


Figure 5.19 The SA distribution of different grades in the experimental groups

5.3.5.4 Variables affecting the serum albumin concentration

It is known that the SA concentration increases in closed eye tears of adults. To investigate if the alertness state of infants effects the concentration a non-parametric equivalent to the one-way ANOVA was used. The regrouping of all infants in open eye, closed eye and crying showed a significant difference on the SA concentration ($p=0.009$, Kruskall-Wallis test). Figure 5.21 shows how closed eye tears have a higher concentration of SA compared to the other states of alertness.

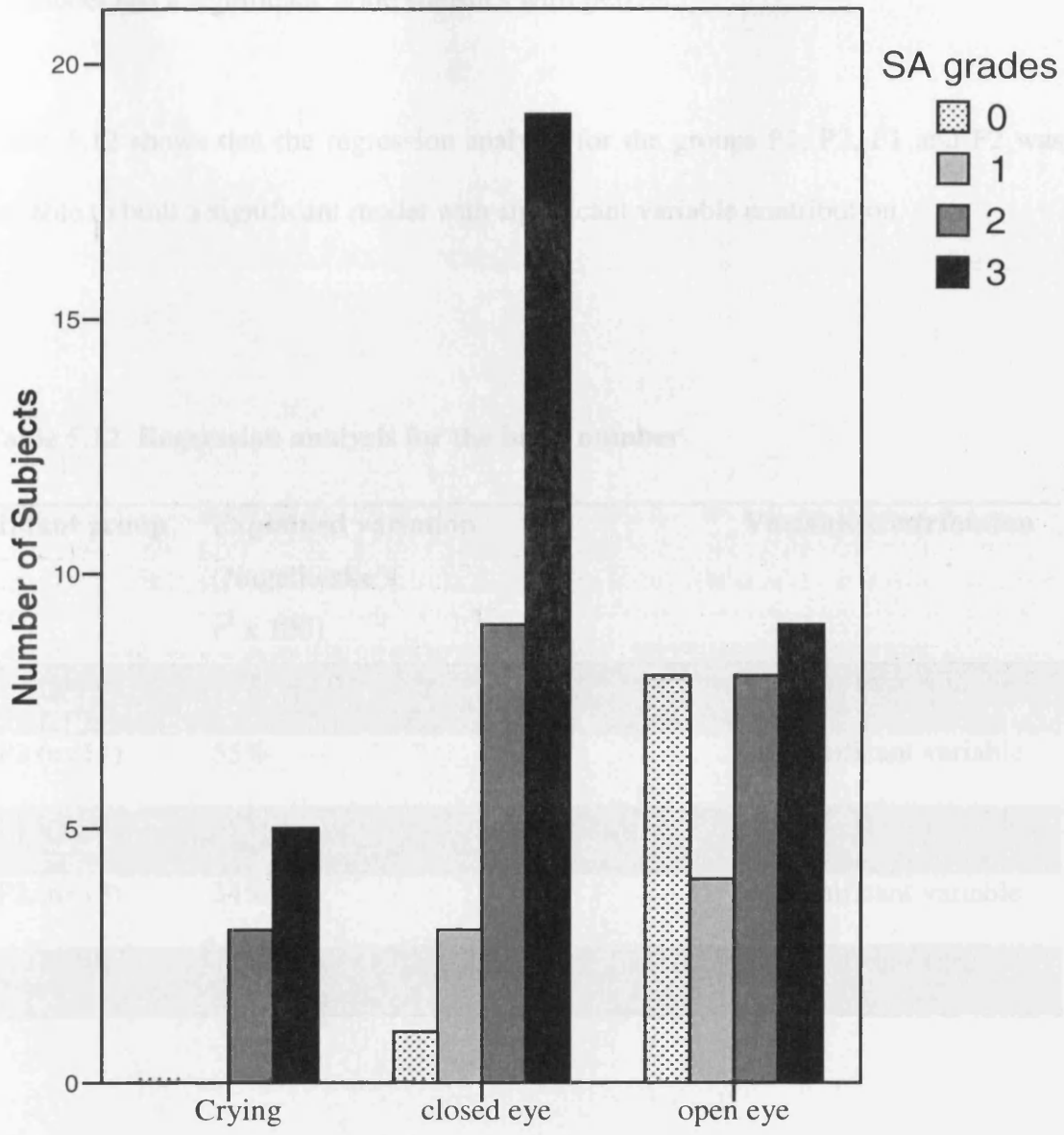


Figure 5.20 The SA distribution of different grades in the alertness states

To examine which other variables such as maturity, gender, volume and protein concentration, could effect the SA distribution, ordinal regression was used. Two stepwise methods for entering the variables were chosen, to duplicate the analysis. The fitted model was significant when $p<0.05$ and the proportion of variation

explained by the model was estimated by Nagelkerke's pseudo r^2. Variables useful to the model had a significant Wald statistics with $p<0.05$.

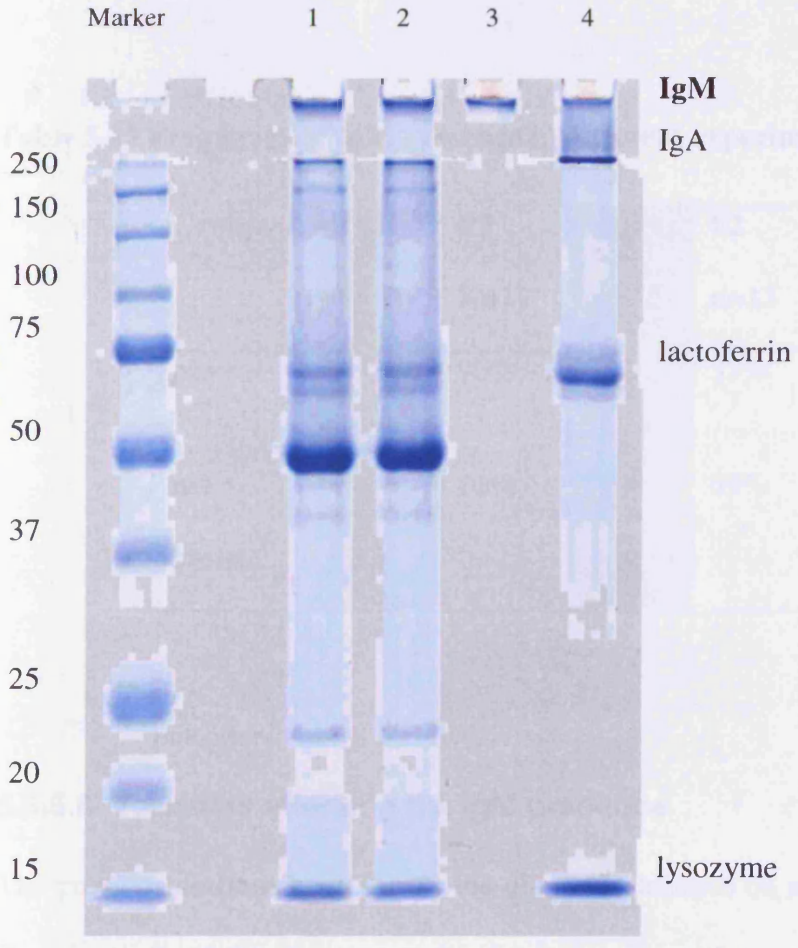
Table 5.12 shows that the regression analysis for the groups P1, P2, F1 and F2 was not able to built a significant model with significant variable contribution.

Table 5.12 Regression analysis for the band number

Infant group	Explained variation (Nagelkerke's r^2 x 100)	p	Variable contribution
P1 (n=23)	37%	0.5	no significant variable
P2 (n=11)	55%	0.4	no significant variable
F1 (n=22)	39%	0.09	no significant variable
F2 (n=13)	34%	0.1	no significant variable
A (n=19)	12%	0.8	no significant variable

5.3.5.5 IgM

The prevalence of the IgM in the uppermost band (Figure 5.17) was identified by western blotting. No band was seen on the top of the SDS-PAGE in the analysis of adults' tears, and they were excluded from the statistical analysis.



Lanes:	
1 and 2:	tears from a premature infant
3:	IgM standard
4:	IgM, IgA, lactoferrin, lysozyme standards

Figure 5.21 Tear protein profile of premature infant tears showing IgM band

The presence of the IgM bands for each experimental group is shown in Table 5.13. Differences were tested by the non-parametric equivalent of the one-way ANOVA and a significant difference was found between the infant groups (p=0.004, Kruskal-Wallis test). The results in Table 5.13 show that IgM is more often present in premature infants.

Table 5.13 Frequency of IgM presence in different experimental groups

Groups	P1 n=23	P2 N=11	F1 N=22	F2 n=13	A n=19
present	83%	90%	55%	31%	0%
not present	17%	10%	45%	69%	100%

5.3.5.6 Variables affecting the IgM presence

The previous sections have shown the effect of alertness on serum albumin and IgA. To investigate if the alertness state of infants affects the presence of IgM, the non-parametric equivalent to the one-way ANOVA was used. The regrouping of all infants in open eye, closed eye and crying showed a significant difference ($p<0.001$, Kruskall-Wallis test). When the effect of alertness was observed in Figure 5.23 the majority of cases with IgM were found in closed eye infants and fewer in open eye infants. IgM was present in all crying infants.

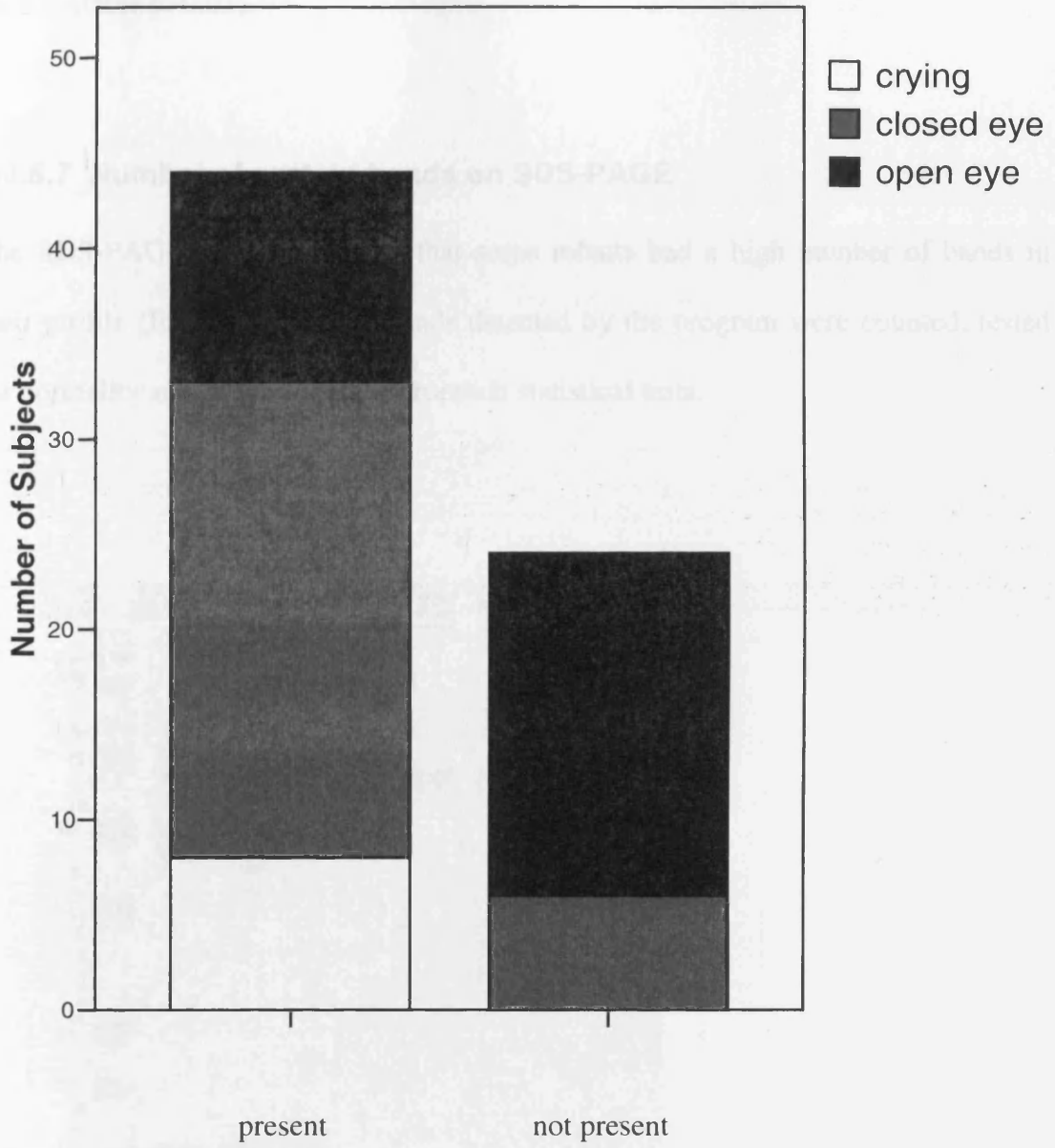


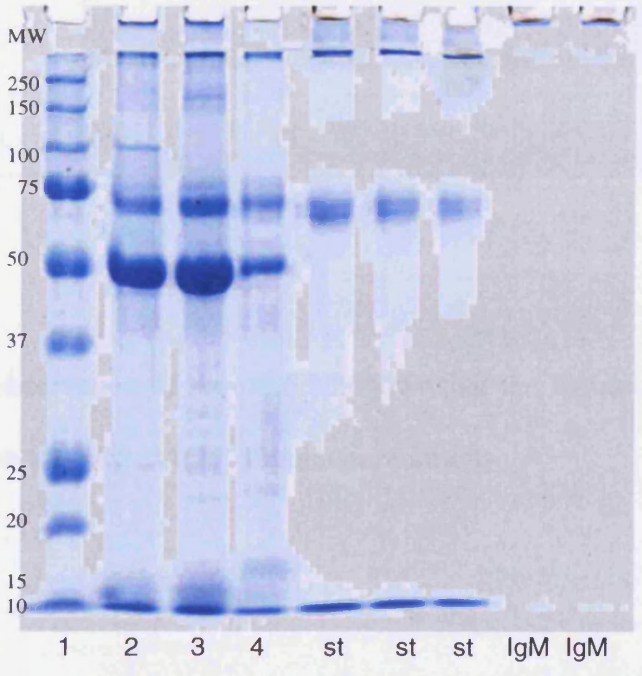
Figure 5.22 The frequency of IgM presence by different alertness states

To examine whether the remaining variables such as maturity, gender, volume size, and protein concentration can predict the IgM presence, logistic regression (binary) for dichotomous dependents was used (see section 5.3.5.1 on lipocalin). Logistic

regression for the groups P1, P2, F1 and F2 was not able to include any of the variables for maturity, gender, volume and protein concentration to build a model (for each variable $p>0.05$).

5.3.5.7 Number of protein bands on SDS-PAGE

The SDS-PAGE analysis showed that some infants had a high number of bands in their profile (Figure 5.24). All bands detected by the program were counted, tested for normality and examined by appropriate statistical tests.



1	Marker
2	premature infant PA
3	premature infant PB
4	premature infant PC
st	standard mix (IgA, lactoferrin, lysozyme)
IgM	IgM standard

Figure 5.23 SDS-PAGE of premature infant tears

A significant difference was found between the experimental groups (p=0.006, ANOVA one-way). Adults showed a significant difference to the premature infants groups P1 and P2 (Table 5.14) and there was no difference between all the infant groups.

Table 5.14 Post hoc (Tukey) test for significant differences in the number of bands between subject groups (NS when p>0.05)

	P1	P2	F1	F2	A
P1		**NS**	**NS**	**NS**	**0.04**
P2	NS		NS	NS	0.02
F1	**NS**	**NS**		**NS**	**NS**
F2	NS	NS	NS		NS
A	**0.04**	**0.02**	**NS**	**NS**	

The observation of Figure 5.25 shows that the highest number of protein bands was seen in the SDS-PAGE of premature infants.

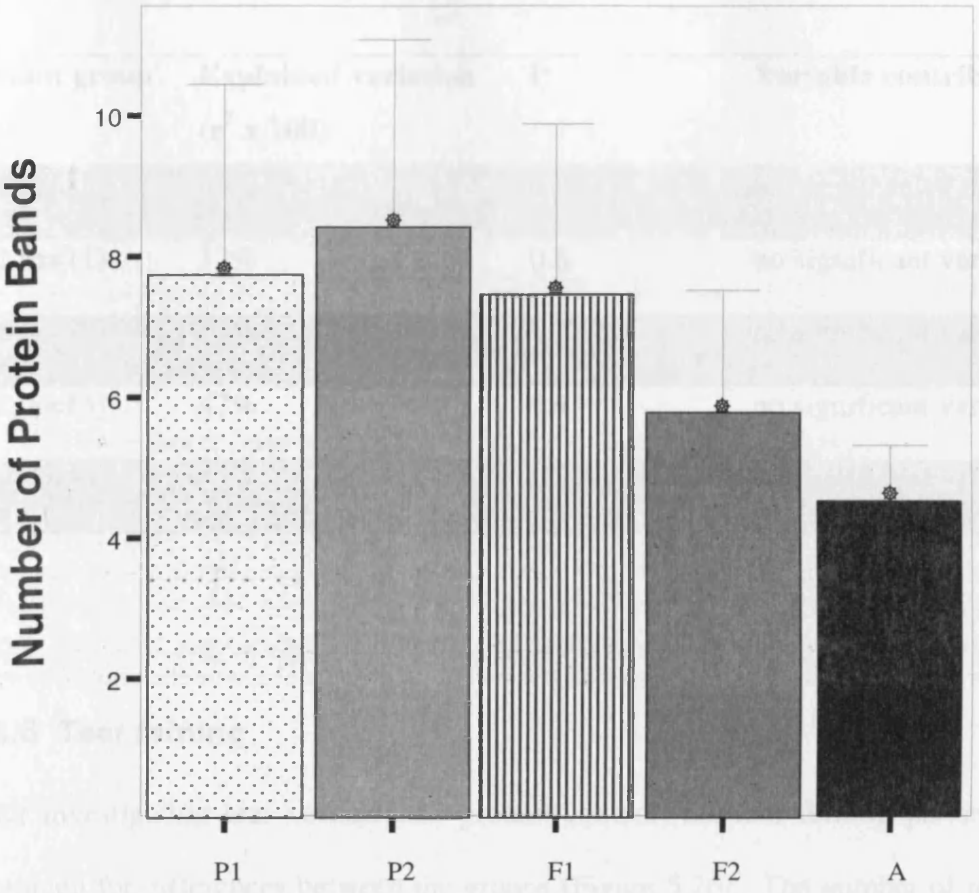


Figure 5.24 The number of protein bands on each lane of the SDS-PAGE in each experimental group (mean±sd)

5.3.5.8 Variables affecting the number of present bands

To test for effects on the variation of band numbers the variables for maturity, alertness, gender, volume size and protein concentration were examined by linear regression analysis. Table 5.15 shows the statistical results. No model was found to explain the variation and none of the variables contributed significantly.

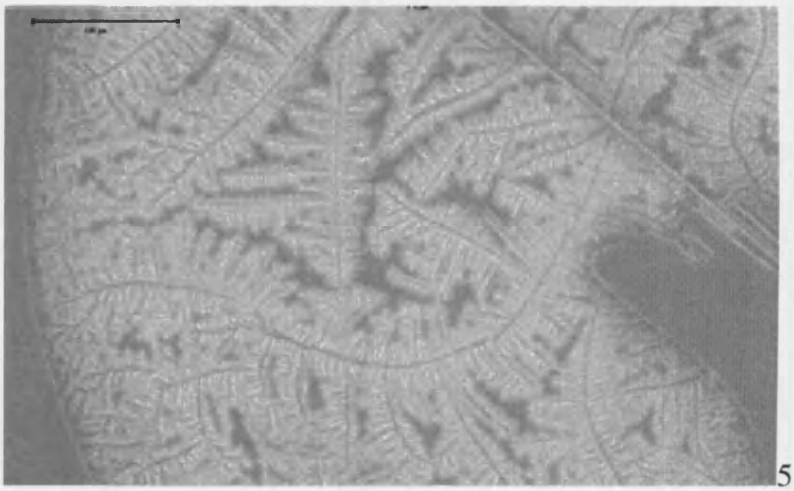
Table 5.15 Regression analysis for the band number

Infant group	Explained variation (r^2 x 100)	P	Variable contribution
P1 (n=23)	40%	0.3	no significant variable
P2 (n=11)	12%	0.8	no significant variable
F1 (n=22)	21%	0.3	no significant variable
F2 (n=13)	47%	0.4	no significant variable
A (n=19)	12%	0.8	no significant variable

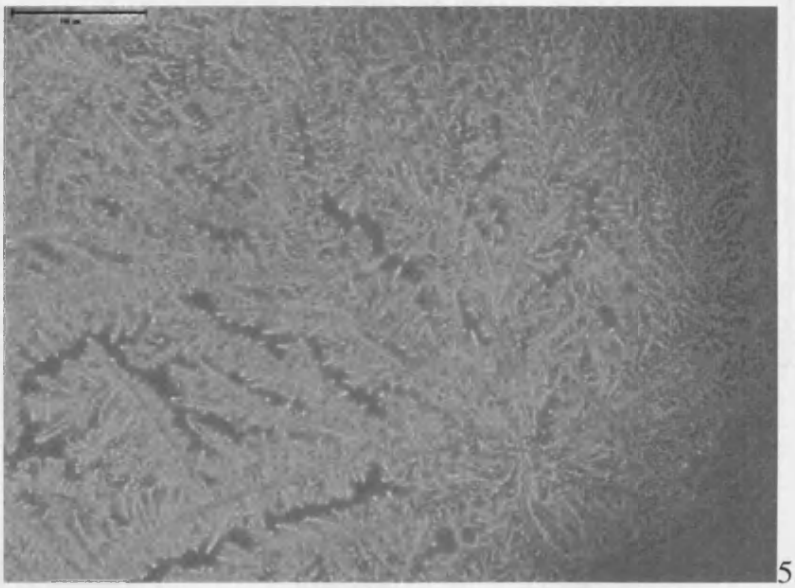
5.3.6 Tear ferning

After investigating tear volume and protein content, the tear ferning patterns were examined for differences between the groups (Figure 5.26). The number of results in each experimental group was dependent on the tear volume size that remained after protein analysis. Tear ferning results consisted of grades and the nonparametric equivalent to the one-way ANOVA was used to test for differences between the infant groups.

5.26 a



5.26 b



5.26 c

Figure 5.25 Ferning patterns show exapmples of tear samples from the experimental groups A (a), P1 (b) and F1 (c). The bar in the left top corner represents a scale of 100µm.

The difference between the grades of the subgroups was not significant when statistically analysed (p=0.1 Kruskal-Wallis test) (Figure 5.27).

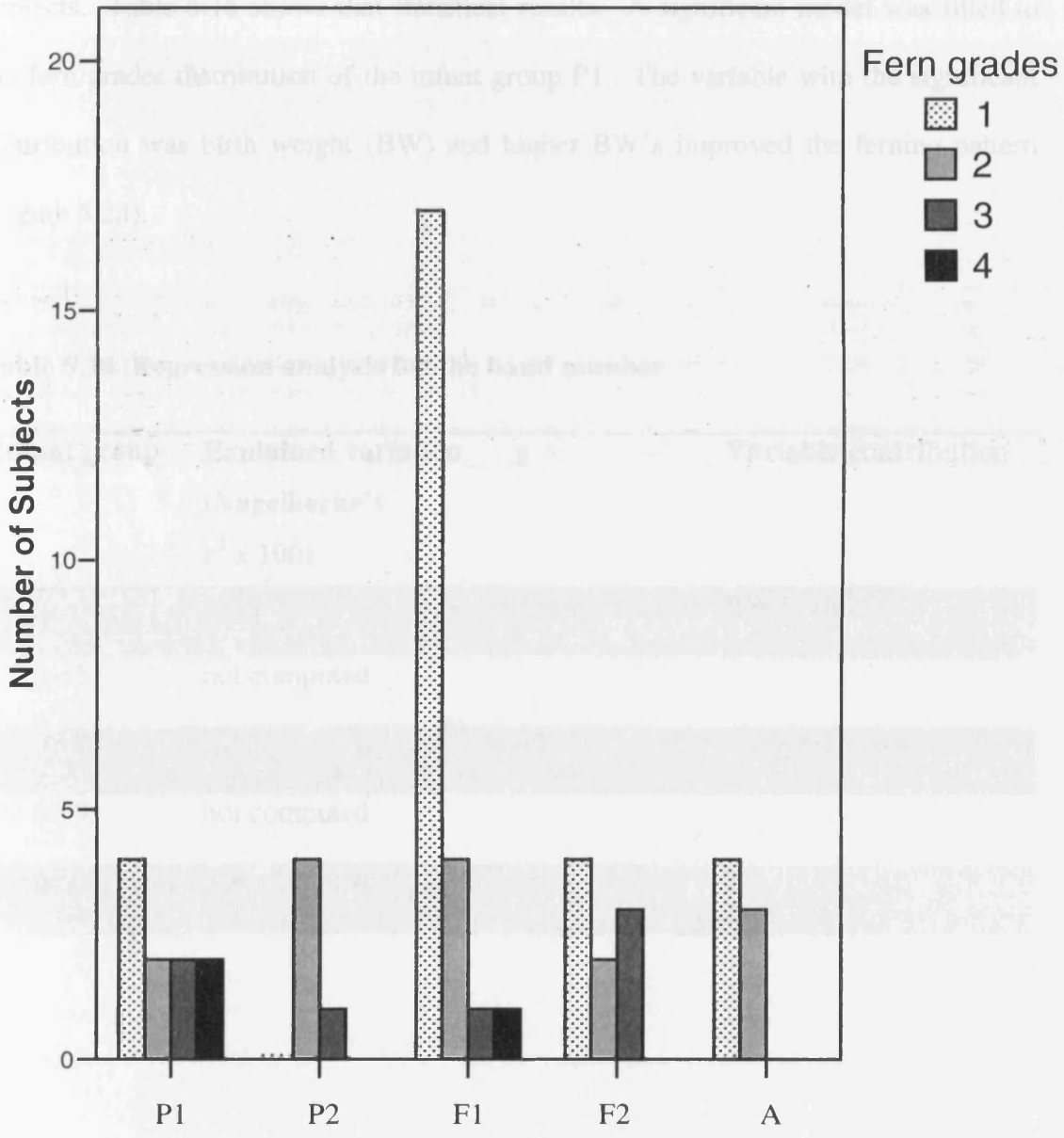


Figure 5.26 Tear fern grades and their frequency for each group

5.3.6.1 Variables affecting the number of fern grades

No difference was found by the alertness of the infants (p=0.4, Kruskal-Wallis test). Ordinal regression analysis was used to test remaining variables for maturity, gender, volume size and protein concentration explaining the result variations for all infant subjects. Table 5.16 shows that statistical results. A significant model was fitted to the fern grades distribution of the infant group P1. The variable with the significant contribution was birth weight (BW) and higher BW's improved the ferning pattern (Figure 5.28).

Table 5.16 Regression analysis for the band number

Infant group	Explained variation (Nagelkerke's r^2 x 100)	p	Variable contribution
P1 (n=10)	93%	<0.001	BW (p=0.02)
P2 (n=5)	not computed		
F1 (n=23)	77%	0.1	no significant variable
F2 (n=9)	not computed		
A (n=7)	not computed		

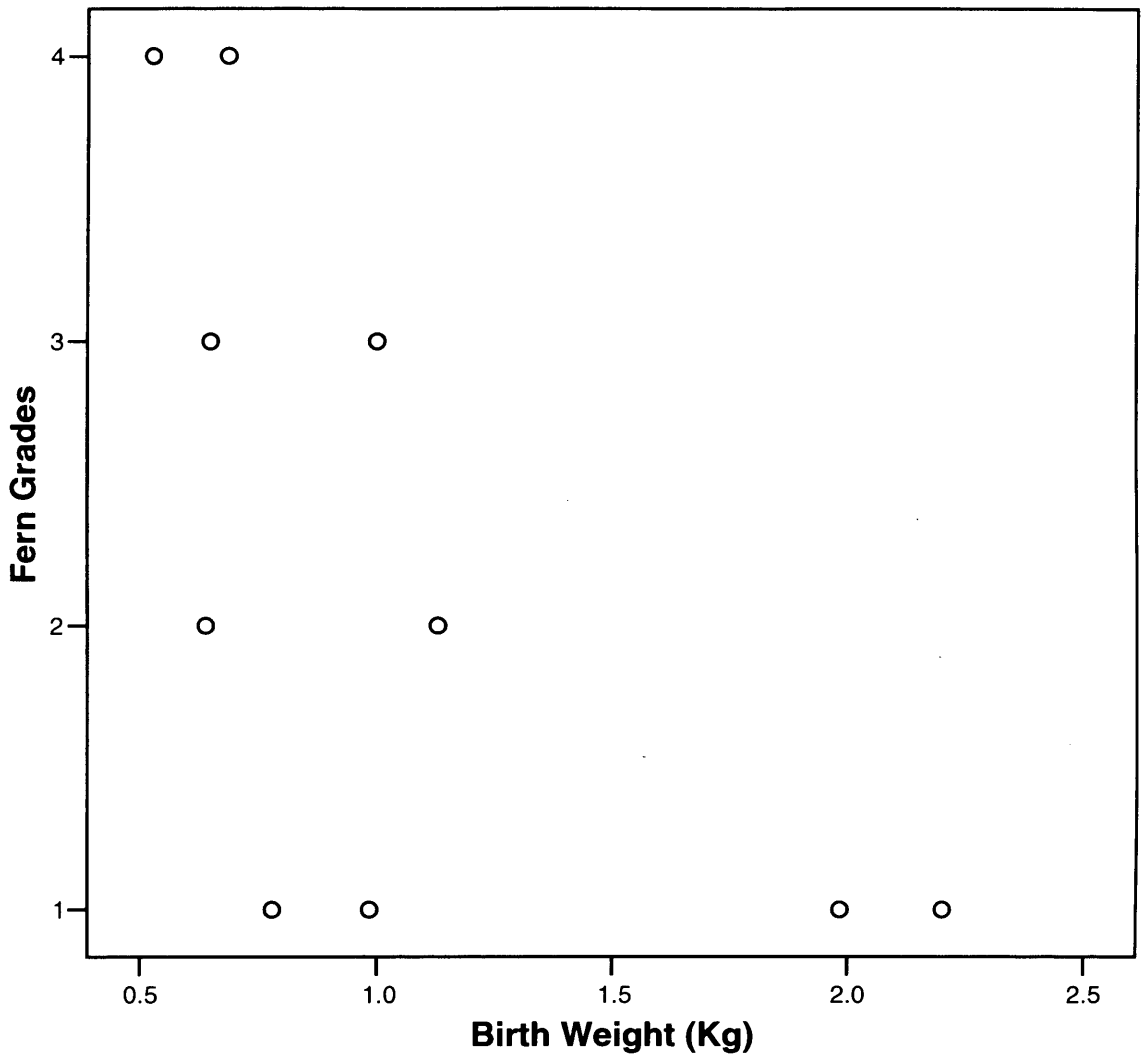


Figure 5.27 Relationship between fern grades and birth weight

5.3.7 RESULTS- Summary

One of the aims for this study was to evaluate the collection technique. Therefore the ability for tear collection was examined by observing the sample size distribution. The results of tear volumes in each experimental group were not normally distributed. When tear collection was repeated all infant's samples had a volume above zero.

The analysis of collected samples showed that there is a difference between the experimental groups in the means of sample volume size. Power analysis (Excel spreadsheet by Dr. Russell Woods, Schepens Eye Institute, Harvard Medical School) showed that sufficient samples were collected for the tear volume analysis. However, the number of results needed for the protein analysis was not practical for this current study[4].

A significant difference between the experimental groups was found when the variables IgA, serum albumin, IgM, and number of present bands were examined. The total protein concentration, lysosyme, lactoferrin, lipocalin, and tear ferning were not affected by the grouping.

The total protein concentration, IgA, serum albumin concentrations and IgM presence were affected by the variable alertness. Lipocalin could not be detected in some of the samples from infants. Finally the majority of infants showed a very good or good tear ferning pattern.

[4] For example: power analysis predicted to find a significant difference between the F1 and A group (difference in the mean protein concentration=2.09, standard deviation=4.5) with 98 samples having sufficient volumes for protein analysis.

When the results in each group were processed by regression analysis, a total number of ten to fifteen results were needed. As this number of samples was not always available, some of the analysis was not computed by the programme. However, to ensure that significant statistical results were accurate, the results were observed and were graphically presented.

5.4 DISCUSSION

5.4.1 Tear sample distribution and collection method

This study found that the cellulose rod is able to collect tears in almost all premature and full term newborn subjects, although with a greater number of small sample volumes. This could be caused by two possible reasons. First, the collection technique was not able to collect tears in some of the subjects. If this assumption is true, the percentage of cases where the technique failed should be similar in each infant group. The second possibility is that some subjects did not have sufficient tear volume on the ocular surface. In the infant groups, the subjects might have had a late onset of tear secretion, as previously reported by Sjögren (Sjogren, 1955). He found in 13% full-term newborns alacrimy, which decreased to only 3% of the subjects in 1-7 weeks.

The missing sample volume in one of the adult subjects may be due to the collection technique, but it is not certain that the same failure rate can also be applied to infants. Generally it was experienced that tear collection in infants was easier than in adults. Since adults fear an object approaching the eye, it is common practise to use experienced subjects. Most tear film studies usually collect from students and members of staff. Infants do not have the same cognitive ability. It was observed that infants co-operate subconsciously unless they were irritable lacking food or sleep as reported by their parents. However, including and excluding the subjects with no tear volumes did not change the statistical test results. The late onset of tear secretion and the ease of tear collection in infant subjects argue that the variation in sample size corresponds with the age rather than with the collection method.

The distribution of tear volume varied in each group, due to a high frequency of absent or low volume in younger premature and full-term newborn infants. This study found that premature and full-term newborn infants had a lower tear volume than adults, but that there was no difference between premature and full-term infant tear volumes. In full-term infants tear volume increased rapidly after birth to a similar level of adult tears.

Many studies have stated that secretion from full-term infants is normal (Apt and Cullen, 1964; Menon, 2000). It was even found that a high percentage of full-term neonates (Akar et al., 2004), 84% of infants up to the age of 10 days and 80% of preterm infants up to the age of 10 days (Patrick, 1974) have normal tearing. In the current study 77.5% of the full-term neonates had enough tears to enable further analysis. Many studies have not found a correlation between full-term tear secretion and maturation factors such as GA, post-conceptional age and birth weight (Patrick, 1974; Spiegler and Mayer, 1993; Menon, 2000; Toker et al., 2002; Akar et al., 2004). Patrick (1974) concluded that no such correlation existed also for premature infants. There are some contradicting reports that consider tear production to be led by maturation. Isenberg et al. (1998) found basal and reflex tears in premature infants to be reduced and reach an adult-like level in full-term infants. Their study showed that basal tears of premature infants correlate to weight and post-conceptional age, whereas reflex tear secretion in full-term infants increases with weight only. Menon (2000) found tear secretion from premature infants to be reduced compared to full-term infants. Akar et al. (2004) measured a 2 month period after birth for premature infant tears to reach normal levels and a four week period for full-term infants. They concluded that post-conceptional age is more important than birth weight. Toker et al.

(2002) reported for premature infants that total tear (basal and reflex) tears correlate with birth weight and post-conceptional age. Their investigation showed that basal tears correlate only with birth weight. There are two reports that showed tear volume differences with weight. The first was Sjögren (1955), who found the highest alacrimy rate in infants between 1000 and 1800gr. The other was Apt and Cullen (1964) who discovered progress in normal tearing with increasing weight of premature infants.

All quoted studies on infant tear secretion have used Schirmer papers to evaluate tear volume and secretion rate. The test was performed for five minutes. In the current study a cellulose rod was used to sample tears. It was used for two minutes. This time limit might vary slightly between individuals. Adults blink more frequently, and in order to prevent reflex tearing, they were allowed to blink every 5-10 seconds. Infants blink less often and the short breaks to allow blinking did not affect the time limit much. However, the variation of collection time between subjects was small and prevention of much less controlled reflex tearing was preferred in the design of tear collection. The cellulose rod collects similar amounts of tear volume in a shorter time as the Schirmer test does for basal tears (i.e. with anaesthetics).

5.4.2 Total protein concentration, specific proteins and sensitivity of comparison between groups

In contrast to tear volume variations, the protein concentration did not show any difference between the subject groups. More defined parameters such as the variables for gender, GA, PCA, BW and actual weight did not alter the protein concentration. In older full-term infants the protein concentration was decreased when they had open

eyes, although the tear volume size did not differ between open and closed eye infants. This means that the tear volume and protein concentration were not related and that no dilution effect with increased volume collection was found in infant tears. In contrast, a decrease in protein concentration was shown for adults in this and previous experiments of this thesis. Samples with higher volume sizes from infants may only be emotional tears and not reflex tears. This is supported by reports on adult emotional tears and the lack of dilution in their protein content (Frey et al., 1981; Kimata, 2004).

The only other study that differentiated between crying and non-crying tears was Apt and Cullen (Apt and Cullen, 1964), but they did not extend the analysis to the protein content. This study found that the total protein alteration shown by the state of alertness is maintained by IgA, IgM and serum albumin. A difference in the tear proteins IgA and serum albumin between closed eye and open eye tears has been shown by other researchers on adult tears (Sack et al., 1992; Sitaramamma et al., 1998b) (Table 5.17). Increase of proteins with reflex tearing and in closed eye tears suggests that they derive from serum (Fukuda and Wang, 2000).

Table 5.17 Comparison of neonate and infant protein concentration with adult-like values, found by the current study and two other investigators. They highlight the effects of closed and open eye, as well as stimulation of tears in open eye.

Age	Total protein (μg/μl)	IgA (μg/μl)	Lactoferrin (μg/μl)	Lysozyme (μg/μl)	SA graded	Reference
premature	12.43±4.32 (Bradford method (1976))	1.18±0.7	1.83±1.03	3.61±2.93	strong bands (2 and 3)	
full-term newborn	10.95±5.51 (Bradford method (1976))	0.6±0.28	1.31±0.59	2.44±1.36	all grades	
full-term	12.93±3.99 (Bradford method (1976))	1.05±0.59	2±0.98	4.1±1.73	not detectable or low (0 and 1)	current study
adults non-stimulated	13.04±3.46 (Bradford method (1976))	1.65±0.83	1.99±0.88	2.68±1.4	not detectable (0)	
adults closed eye	18±7.4 (Lowry Method (1951))	8.4±2.32		1.1±0.76		
adults non-stimulated	9±6.6 (Lowry Method (1951))	0.85±0.24		0.06±0.02		Sack et al. (1992)
adults stimulated	6±2.5 (Lowry Method (1951))	0.23±0.05		0.02±0.01		
adults stimulated	6.05±1.58 (Bradford method (1976))		2.73±0.82	2.46±0.44	0.021±0.028 (μg/μl)	Ng et al. (2000)

Table 5.17 shows the variation in total and major protein concentration of open, closed and reflex tears found by other studies and compares it to the mean concentrations found in the current study. Infant tears show some differences to adults' closed and open eye tear models. For example, although IgA was affected by the alertness state of the infant, its concentration in neonates was still lower than in adults and increased during the first four months of life. The closer investigation of major proteins showed that some proteins are distributed in different concentrations in the experimental groups:

- IgA

The IgA concentration was lowest in full-term newborn infants. It showed the same variation between the different alertness states as the total protein concentration. In older full-term infants the IgA concentration was a major part of the protein concentration since it affected the total protein increase. On the other hand, IgA decreased with post-natal age. That could be related to the increase in tear volume and a slower maturation rate of IgA with age. Only one investigator has measured the total protein concentration of infants and found very low concentrations (Patrick, 1974). Some investigators even believed that infants are essentially IgA free (Mull, Peters and Nichols, 1970; Watson et al., 1985). These reports are not recent (Table 5.18) and they may have used less sensitive assays. In this study, small sample sizes were taken and the IgA findings may indicate that there was no dilution effect as it has been described in large tear volumes (Fullard and Tucker, 1991).

Table 5.18 Literature review of neonate and infant protein levels, found by different investigators: The presented studies are very old and may lack a general understanding of tear film by comparing tear's proteins to serums' and ignoring lacrimal proteins (Allerhand et al.); some looked only at a small number of subjects (Bonavida et al., ; Patrick), collected tears after inducing crying (Allerhand et al., ; Etches et al.) and analysed their results insuffiently (Mull et al., ; Etches et al.).

Year	Reference	Number of subjects	Protein content
1963	(Allerhand et al.)	22 full-term (<36hrs)	Serum-like pattern with lysozyme
		26 full-term (36-148 hrs)	older infant's pattern were between young and controls
		21 premature (1week to 30days)	premature infant protein patterns reach older full-term in one months
1969	(Bonavida et al.)	4 full-term	lysozyme 0.7-0.8 (μg/μl)
1970	(Mull et al.)	131 full-term	IgA (0-7days) no IgA (8-14 days) 11% prevalence (61-90 days) 75% prevalence
			no albumin found at any age
1974	(Patrick)	3 for total protein	total protein 4.2, 8.8, 13.2 (μg/μl)
		16 for IgA	IgA 2-7% of adult levels (adults: 1.5-3.3 μg/μl)
		2 subjects for lysozyme	lysozyme 1.1, 1.6 (μg/μl)
		full-term and preterm (0-10days)	
1978	(Watson, Reyes and McMurray)	27 children (2-5 yrs)	total protein 13.9±1.8 μg/μl
			secretory IgA 0.49±0.08 μg/μl
			lysozyme 1.03±0.18μg/μl
			SA 0.34±0.08μg/μl
1979	(Etches et al.)	20 full-term	lysozyme 1.118 μg/μl
		17 premature	significantly less diameter zone of lysis (mm) in premature infants

- Lactoferrin

The lactoferrin concentration did not differ between infants and adults, or between any state of alertness. Maturation and gender did not contribute to its variation. No other study has yet looked at the lactoferrin concentrations in infants.

- Lysozyme

Only the lysozyme concentration did not show any difference between the groups, nor was any other variable found to explain the lysozyme variation among infant subjects. Lysozyme may be the major protein of the newborn full-term infants, as results in group F1 suggest. There was no relationship to tear volume secretion. In adults the lack of this relationship (Fullard and Tucker, 1991) was explained by the regulation of its production in the lacrimal gland (Dartt, 1989). Lysozyme has been the most widely studied protein in infants and children (Table 5.18); its concentration comprised a wide range in the literature.

- Lipocalin

Lipocalin has its source in the lacrimal and the same regulation of production as lysozyme (Dartt, 1989). It was absent in some infants. One infant with absent lipocalin was followed up at 4 month of age and still no lipocalin could be found by Western blotting. In premature infants (P1) the probability of lipocalin absence was higher in small samples. Lipocalin has lipid-binding and carrying properties and may need further investigation since it contributes to the spread of the superficial lipid layer (Bron and Tiffany, 1998; Zhao and Wollmer, 1998; Glasson, Stapleton and Willcox, 2002; Miano et al., 2002). Glasson et al. (2002) showed that increased levels of lipocalin were correlated with contact lens intolerance. Full-term infants

have been shown to have a high quality of lipid layer that improves blink rates (Lawrenson et al., 2003). This shows how important it is to investigate the onset of lipocalin secretion, its concentration and effects on infant lipid film.

- Serum albumin

Serum albumin concentration was highest in the premature infants and not measurable in adults. It increased when infants were crying or had closed eyes. Serum albumin has not been measured in infants before. Concentrations found for children and adults in this and other studies (Table 5.17) were very low.

- IgM

Initially IgM was not expected in infant tears but a band on the top of the gel proved to contain this protein. IgM has been found in very small concentrations in adult tears by Fullard and Snyder (Fullard and Snyder, 1990) using HPLC. In infants it may reach the tear fluid via ocular surface vessels (Franklin, Prendergast and Silverstein, 1979), with tear levels decreasing with the diluting effect of an increased tear flow rate. It has previously been found only in infants in the presence of trachoma eye infection (Mull et al., 1970). Another possibility to explain its presence, is its production in the lacrimal gland suggested by Kuizenga et al. (Kuizenga et al., 1990). They found in a study with IgA deficient adults that IgM could have a compensatory role. Its presence was more often found in infants crying and with closed eyes. This may indicate local control similar to the variation in the IgA concentration.

- Other bands

Many other bands were observed on the SDS-PAGE of infants. They were counted after densitometry and it was shown that premature infants have the highest number of bands present. These bands could be unknown proteins, serum proteins leaking into the tears or degraded proteins that show more than one band.

In this study tear ferning was determined for all experimental groups. Only full-term newborn infants had a tear film showing the same ferning grading as the adults. This difference shows that neonate tear contents are balanced shortly after birth. Developmental changes of the infants and their environment may disturb this balance. Puderbach and Stolze (1991) used the technique of tear ferning on a group of children with a wider range than in this study. They found in most of their subjects good grades (17% had type I, and 63% type II ferning, according to Rolando's grading system).

In conclusion, tears were collected by a new collection method, the cellulose rod from very young premature and full term infants. It proved possible to take tears without disturbing the infant. It was also sensitive to differences between the experimental groups mentioned by the literature and differences in secretion of tears. Overall tears were graded as normal by tear ferning. Many interesting differences in tear protein profile were found that show similar protein control mechanisms as in adults. These mechanisms, such as for open and closed eye and for maturation of secretion, need to be studied in more detail.

6 Tear film collection from infants and children with contact lenses

6.1 INTRODUCTION AND AIMS

A preliminary tear film collection study was carried out including infants and children who wore contact lenses. The intention of this tear collection study was:

- To assess the performance of the cellulose rod in a wider age range, from young infants to older children
- To assess the flexibility of sample collection in a clinical setting and transport from a test location to the processing lab
- To collect preliminary results of the tear film of paediatric contact lens wearers

This study was intended to extend tear collection to other subject groups and settings. Another group of subjects regarded as difficult for sample collection are young children. In contrast to infants that were not aware of the tear collection, young children have to consciously participate. There may be difficulties in co-operation when a capillary tube is used. The fear of a sharp object approaching the eye may lead to squeezing the eyes and this is counter-productive for non-stimulated tear film collection.

A clinician, who does not use the tube as frequently as a specialised researcher, might also be afraid of using a capillary tube. This may be one of the reasons that tear collection and analysis is not as popular as other clinical methods for assessing the

tear film (Korb, 2000; Turner et al., 2005). There is a need for this study to confirm the ease of the cellulose rod tear collection technique use by a clinician.

Finally, this study was aiming at paediatric contact lens wearers. One complication for paediatric contact lens wear is the increased attachment of tear proteins to the contact lens by the age of three years (de Brabander et al., 2002). This has not been further evaluated in the paediatric contact lens wearers. Proving that the cellulose rod can be applied to this subject group will enable future studies of the tear film and contact lens tolareance.

6.2 METHOD AND MATERIALS

6.2.1 Recruitment

Collaboration was established with Prof. A. Jonathan Jackson at Belfast Royal Victoria Hospital (Department of Ophthalmology). Prof. Jackson recruited from his cohort of contact lens wearing infants and children when they presented for their contact lens review assessment. He was also responsible for tear collection and sample storage until delivery.

6.2.2 Ethical approval

Ethical approval was obtained from the Central and Local Office for Research Ethics Committees (Reference number 04/WSE02/125; date: 8/11/2004) and from the local Research and Development office (R&D) in Belfast for contact lens wearing infants and children.

6.2.3 Subject recruitment

Tears from the contact lens wearing children were collected during their contact lens assessment. During recruitment, parents received an information sheet similar to the previous study but adapted for the contact lens wearing patients. Details of tear collection and implications for contact lens wear were discussed with them and an opportunity given for questions before written consent was obtained. Parents were usually present when tears were collected.

Some of the contact lens wearing children already had undergone an ophthalmic investigation for glaucoma. If this ophthalmic examination had been requested under anaesthesia (possibly up to the age of 2.5years), appropriate samples were collected in the theatre when the child was asleep. It was noted if the contact lens was *in situ* and the collection of tear samples on these occasions did not involve unnecessary removal, insertion or handling of the extended wear contact lens did not occur. Further information about contact lens type, history and wear scheme, general health, therapeutic drugs, and the ease of collection were obtained. Where children were able to understand and were used to contact lens assessments, they were asked to cooperate.

6.2.4 Subject's history

Eleven infants and children were recruited. Even though all subjects presented for a contact lens assessment, each subject was unique in its history and sample collection results. For presentation in this chapter their names were masked by codes starting with B and second alphabetic digit A-K in the order of recruitment. Prof. Jackson was provided with a questionnaire (Appendix) to note information about each subjects

tear collection, such as duration of the collection, state of the infant, and any eye drops instilled before collection. This form also asked for information about the patient's cataract removal, medication, and contact lens type and wear.

Table 6.1 shows the range of subject's age, gender and whether the eye was aphakic. Most of the paediatric subjects (five male and six female) had undergone congenital cataract surgery (usually 1st-15th week) and were aphakic either in both eyes or in one eye only. A tear sample was taken from the contact lens wearing eye and, if the appointment time allowed, a second tear sample was taken from the non-contact lens wearing eye.

Table 6.1 Subjects age, gender and presence of aphakia

Subjects	Age in years	Gender	Aphakia
BA	1.5	male	Yes
BB	1.25	female	Yes
BC	8.8	male	Yes
BD	6.8	female	Yes
BE	5.3	female	Yes
BF	6.7	male	Yes
BG	2.5	male	Yes
BH	4.5	female	Yes
BI	2.2	female	No
BJ right eye	0.26	female	Yes
BJ left eye			No
BK	0.24	male	Yes

The youngest subjects were two infants, 11.72 weeks and 12.43 weeks old. Their congenital cataract was removed 4 weeks after birth and had healed long before the infants presented. All other children were between 1.5 and 9 years old.

A great diversity of contact lens types was noted. Two subjects were soft contact lenses wearers, five were RGP wearers, one child was fitted with silicone hydrogel and three others had silicone lenses. Although all subjects presented for contact lens review assessments, four subjects did not have the lens *in situ* when they presented and their tears were collected. Apart from problems caused by contact lens fit and loss, it was noted that four subjects had recurrent episodes of red eye since the first contact lens fitting.

Five subjects were using medications that could have had an effect on the tear film and its collection. For example, three subjects (BC, BD, BH) were regularly taking glaucoma medications in the form of eye drops and BG used artificial tears. BJ received atropine in his non-aphakic eye. Two subjects had systemic treatments, antihistamines (BD) and treatment for skin eczema (BF). However, these medications were instilled or taken at least two hours before the subject presented for the study.

Eye drops, such as anaesthetics and fluorescein were used before the glaucoma screening and mydriatics were used just before the tear collection for BD and BH.

6.2.5 Tear collection

Tears were collected by the cellulose rod, using the standard protocol of tear collection (Chapter 4) and extraction (Chapter 2). Briefly, prior to use, the rods were

disinfected by 5 minutes exposure to UV light. Tears were collected with flexible cellulose rods by gently touching the tear meniscus at the lower lid. Tear collection was rapid and extended only to a maximum of two minutes.

After collection the cellulose rod was placed into an Eppendorf tube (0.5µl) and sealed. Each Eppendorf tube containing the sample was kept at -4°C until the end of the clinic day and transferred to a freezer at -20°C. For the delivery from Belfast to Cardiff, they were packaged with ice-blocks in polystyrene boxes. A 24hr delivery service from DHL ensured that the samples arrived frozen. Samples were analysed the same day of arrival. The duration between collection and analysing did not exceed one week. Storage at -20°C had been previously investigated (Chapter 4) and it ensured that samples were not affected by the delay before analysis. The extraction protocol was used as described in Chapter 3.

6.2.6 Recording and Analysis

Tear samples were analysed by their volume size, protein content and tear ferning. The protocol is described in Chapter 5.

6.3 RESULTS

6.3.1 Tear volume and protein analysis

Tear collection was not always possible for the duration of two minutes as intended. On average the collection was stopped after one minute and seven seconds (±36 seconds). Among the collected samples were emotional tears from five children, two

samples with reflex tears and two subjects were asleep under anaesthesia. In four children the eye lids were not held open during collection.

There was sufficient volume in all samples to measure the total tear protein concentration and analyse the protein profile in all infants.

Table 6.2 shows the tear volume size, total protein concentration and the analysis of major tear proteins of each paediatric subject. For an easy and quick comparison to adult concentration of IgA, lactoferrin and lysozyme, these protein bands were graded 0= absent, 1= weak, 2= when within ± 20% of the mean for adults, 3= stronger than 20% of the average for adults.

The adult total and major protein concentrations described by their mean±sd from the previous Chapter are shown at the end of Table 6.2. Observation of the results from infants and children showed that total protein concentrations vary, but none are below or above the normal limits measured for adults. IgM and lipocalin were not always present on the SDS-PAGE. IgA was mostly decreased, while lactoferrin showed a high variability. The lysozyme concentration of children had similar concentrations to adult lysozyme.

Table 6.2 Protein profile for individual contact lens wearing infants and children. Results include the average of adults tear volume and protein profile from Chapter 5. Four subjects (underlined) have had recurrent red eye problems. Last two subjects are young infants. IgA, lactoferrin, SA and lysozyme were graded according to the thickness and darkness of the band. 0 is the band is absent, 1=weak, 2=medium, 3=strong. IgM and lipocalin were observed and if not-present it was noted.

Subjects (Age/years)	Alertness	Volume (µl)	Total protein concentration (µg/µl)	IgM	IgA	lactoferrin	SA	Lipocalin	lysozyme
BA	crying	20.00	10.40	not-present	1	1	0	present	2
BB	crying	10.00	9.30	present	1	1	1	not-present	2
BC	tearing	20.00	10.60	present	1	1	1	not-present	1
BD	closed eye	4.00	10.00	present	1	3	3	present	3
BE	open eye	1.50	10.40	present	3	1	2	present	2
BF	tearing	5.50	12.10	not-present	1	2	1	present	2
BG	crying	17.00	15.10	not-present	2	2	2	not-present	2
BH	crying	7.00	12.50	not-present	1	3	2	present	2
BI	crying	20.00	10.65	not-present	1	1	2	not-present	2
BJ right eye	open eye	3.50	9.50	not-present	1	2	1	not-present	3
BJ left eye	open eye	2.50	12.00	not-present	1	2	1	not-present	3
BK	closed eye	1.10	16.70	present	2	2	1	present	3
Adults	open eye	8.98±8.54	13.04±3.46	not-present	2	2	2	present	2

One infant (BJ) and one child (BA) were followed up a second time. BJ presented after two weeks. BA's tears were collected at 1.5 years of age and again after five months. The results from the first and second collection are shown in Table 6.3.

The second collection from BA resulted in a lower tear volume, although this child was crying. The protein concentration was slightly lower than in the first sample. It is not known if this subject cried because he didn't like the tear collection or for any other reason. It was also not noted for how long the tear collection was carried out. The effect on the protein concentration was similar and no difference in the protein profile was detected between the first and second sample.

The samples from BJ's right and left eye had a higher volume at the second collection and showed an overall dilution effect of the protein concentrations. However, they were similar between the right and left eyes.

Table 6.3 The results from repeated tear collection

Subjects (Age/years)	First or second collection	Alertness	Volume (µl)	Total protein concentration (µg/µl)	IgM	IgA	lactoferrin	SA	Lipocalin	lysozyme
BA	first	crying	20.00	10.40	not-present	1	1	0	present	2
BA	second	crying	14	7.2	not-present	1	1	0	present	2
BJ right eye	first	open eye	3.50	9.50	not-present	1	2	1	not-present	3
BJ left eye	first	open eye	2.50	12.00	not-present	1	2	1	not-present	3
BJ right eye	second	open eye	7.3	10.5	not-present	0	1	0	not-present	2
BJ left eye	second	open eye	8.2	9.10	not-present	0	1	0	not-present	2

6.3.2 Tear ferning

Due to sufficient sample sizes, the tear ferning patterns of 9 children were analysed according to Rolando et al. (Rolando, 1984). The viewed samples showed a very dense tear fern pattern as shown in Figure 6.1. These examples are fernings from the infant subjects BK and BJ. This ferning corresponded to grade I. In two subject results, the pattern was very dense (Figure 6.2). The pattern consisted of tight small ferns close to each other. However, this type of tear ferning does not resemble grade III or IV. The fernings contained a lot of dots or blobs that could be cells or lipids. In all subjects tear ferning established a healthy state in the tears but illustrated a need for further investigation to find the source of the dense pattern.

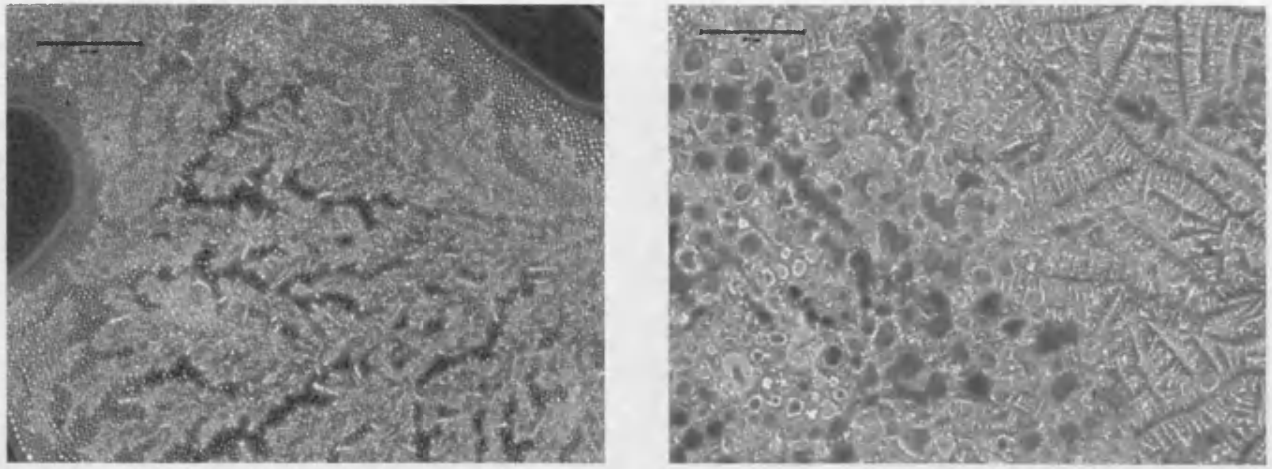


Figure 6.1 Tear fern of BK (left) and BJ (right)

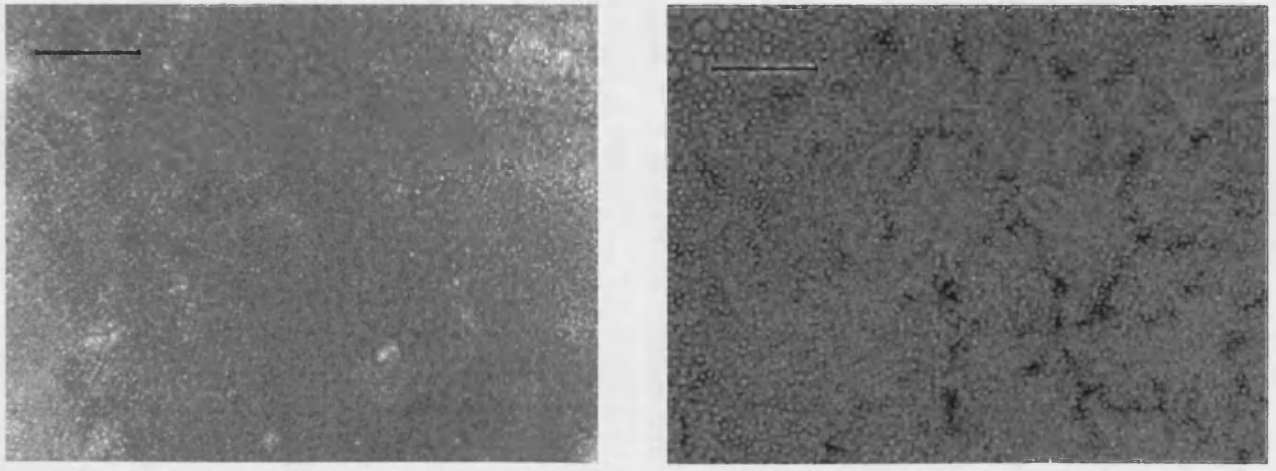


Figure 6.2 Tear fern of BG (left) and BH (right)

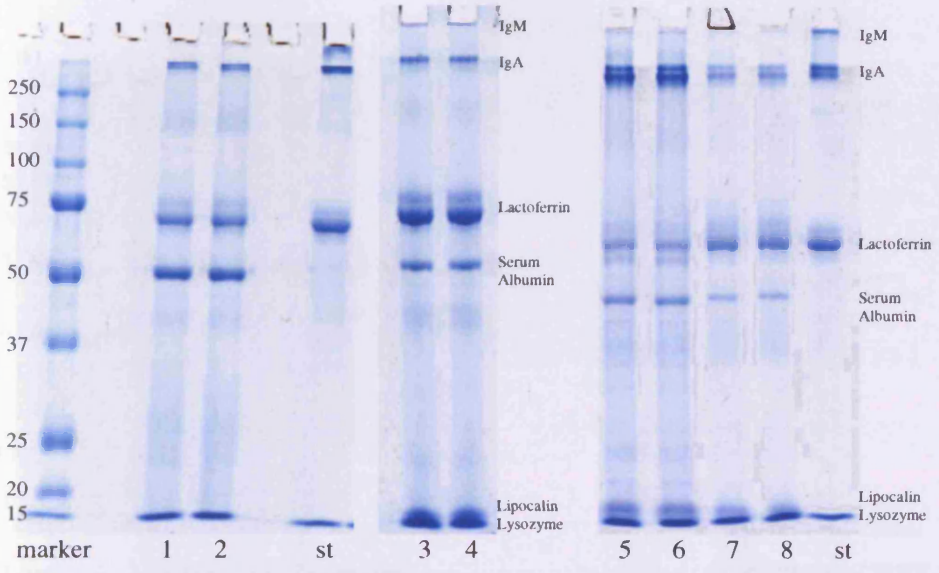
6.4 DISCUSSION

In this study, subjects were recruited in a clinical environment under the condition that they were paediatric contact lens wearers. When comparing between a wide age range, tear collection in children proved more difficult than in young infants. It was reported that some children cried during collection and collection time was in 8 subjects 1 min or below. Despite this difficulty, sufficient sample volumes were collected from all subjects. It was possible to ensure safe delivery of samples to the processing lab within one week. This study was able to demonstrate that tears can be successfully collected from contact lens wearing children and infants by a clinician in a hospital environment and that they can be safely transported for analysis.

Many variations between the subjects were found, such as different contact lens wear or problems associated with the contact lens wear, but these patients were not unusual. There are many reports on visual complications (Zetterstrom, Lundvall and Kugelberg, 2005) and glaucoma (Lundvall and Zetterstrom, 1999; Zetterstrom et al., 2005). According to the literature, infants and children seem to tolerate extended contact lens wear (Gurland, 1979). However, the patient records of four subjects reported episodes of red eye, caused by inflammation, but at the time of tear collection these children had a healthy ocular surface. Inflammation may be caused by children not handling the contact lenses appropriately or a child's susceptibility when wearing a contact lens. For example, one of the four children with a history of red eye (BE) had a different protein profile (Figure 6.3). A higher number of protein bands was found and the lactoferrin concentration was decreased. Lactoferrin has an anti-inflammatory role in the tear film (Legrand et al., 2005). The number of protein bands has also been reported to change in diabetes and dry eye (Grus et al., 2002;

Grus et al., 2005). For clinical purposes this tear sample can be analysed and compared to a sample collected when an infection reoccurs.

Figure 6.3 SDS-PAGE with tears of four subjects with a history of red eye



Lanes are:

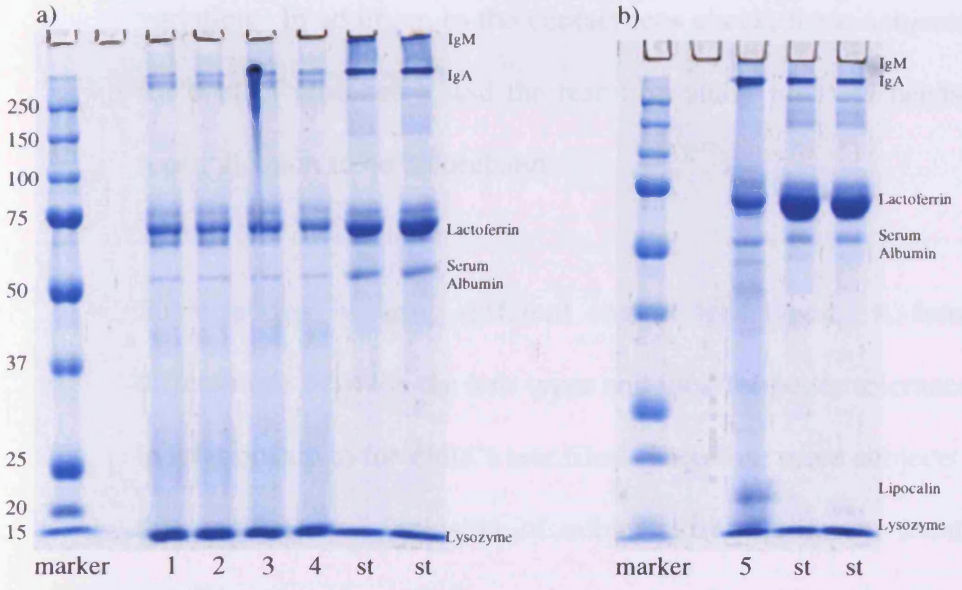
1, 2:	BG
3, 4:	BH
5, 6:	BE
7, 8:	BF

st : standards

Although each subject was handled differently, the protein profile was sensitive enough to show the differences. A difference was, for example, noticed in the comparison of the results from the two infant tear samples (Figure 6.4). When BJ's tears were collected the eyes were gently hold open and BJ stayed alert without noticing the cellulose rod. In contrast BK's tears were collected after Perkins tonometry. BK's tear profile showed many bands that could be caused by leakage

from the ocular surface. The serum albumin band was also denser than observed in adults or in BJ's tears.

Figure 6.4 Infant subject tears on SDS-PAGE: the tear profiles of two infants with approximately the same age were showing differences



Lanes are:
1, 2: right eye tears BJ
3, 4: left eye tears BJ

st : standards

5 : tears from BK

Since an undisturbed contact lens tolerance is essential for a child's visual development more thorough investigation of the tear film, contact lens induced inflammation and its causes are needed. However, in this preliminary study the following difficulties were experienced with the analysis of the results:

- The variety in subjects was observed and needs to be considered for future studies. That means grouping and appropriate exclusion criteria's are needed.

For example, a high number of the aphakic children in this study have a risk of developing glaucoma and some are taking glaucoma medications. The effect of these medications on the paediatric contact lens wearer's ocular surface and tear film needs to be further evaluated. Subjects with other medications, such as antihistamines, also need to be excluded to avoid additional uncontrolled variation. In addition, to the contact lens check, these subjects are often seen for contact tonometry, and the tear film study protocol needs to arrange the tear collection to be beforehand.

- Subjects were wearing different contact lens types. A future study could differentiate between the lens types and look for better tolerance and prognosis in relationship to the child's tear film. Therefore more subjects are needed and that requires the inclusion of subjects from different locations. This is possible since the cellulose rod proved to be an excellent device for a non-experienced clinician and can be easily delivered over a long distance.

- Despite different collection times sufficient sample volumes were collected. For future studies the collection time could be further decreased.

In conclusion this preliminary study applied a collection method developed for research on infant and children tears in an actual clinical situation. Tear collection, delivery and analysis were easy, inexpensive and sensitive to variations. Tears from patients can hence be analysed after each routine assessment and may be used for research or clinical follow-up. This way, patients with recurrent inflammations may benefit from a regular analysis of their tear film.

7 General discussion

In this chapter the main findings of this thesis and their clinical implications are discussed. Some aspects of the study design are reviewed and suggestions for future management and research are made.

7.1 SUMMARY OF THE RESULTS

- Optimal extraction protocol (Chapter 2)

For extraction, different centrifugation forces, times and temperatures were tested and the optimal protocol was designed to ensure maximum sample extraction from absorbent materials.

- Ideal collection material (Chapter 2 and 4)

Optimal fluid extraction was found when the cellulose rod was tested using the established protocol. Effects of storage, sterilisation and suitability for protein analysis were also considered.

The cellulose rod was then applied to collect tear samples from adults. The collection material and technique proved to collect sufficient tear samples and did not alter the tear protein concentration. It did not provoke serum leakage from the ocular surface and could collect a higher volume of tears when they were stimulated. When compared to the standard tear collection technique, the capillary tube, no difference in the mean total and major protein concentrations was found.

- The cellulose rod for neonates and infants (Chapter 5)

The majority of the tear collections contained a sample. The success rate improved with higher post-conceptional age and can be related to the maturation rate of the secretion system.

- Infant tear protein content (Chapter 5)

In the collected infant samples, no dilution effect of the protein concentration was found with increased volume size. In conclusion, there may be no reflex tearing in infants although some large tear volumes were collected. The protein secretion is rather influenced by open, closed and crying eye tears. The lacrimal gland protein lipocalin, that has a similar secretion control as lactoferrin and lysozyme, was not always present. In premature infants it was only found with large sample volumes and this may indicate the maturation of the lacrimal tear secretion. Other differences to adult tears were found, such as the presence of many more bands on the SDS-PAGE correlating to the gestational age. However overall, tears showed the fern ing pattern of a healthy tear film.

- Clinical application (Chapter 6)

Tears were successfully collected and analysed from paediatric contact lens wearers. Tear collection in a clinical setting and the transport of cellulose rod samples to the laboratory was successfully carried out. To study paediatric contact lens wear, tear collection can be extended to more than one location and can be delivered to the analysis site.

7.1.1 Implication on infant tear film research

The main aim of this thesis was to enable tear collection in infants with an appropriate method. The summary shows that not only this aim was met, but by collecting and analysing infant tear proteins, this thesis is superior to the recent infant aqueous research that only described infant tear secretion onset and rate (Isenberg et al., 1998; Menon, 2000; Toker et al., 2002; Akar et al., 2004; Rohatgi et al., 2005).

Proteins are involved in the regulation of different tear layers, e.g. they can be found in the mucous, such as IgA (Mestecky et al., 1978) or be involved in the lipid layer, such as lipocalin (Miano et al., 2002). Being able to collect infant tears opens the door to research that may explain the novel findings of a superior infant tear lipid layer (Kaercher et al., 1994; Isenberg et al., 2003; Lawrenson et al., 2005) or may be the start to examining the infant mucous layer.

7.1.2 Clinical implication for infants

Infants, particularly premature infants, are very susceptible to infections that occur mainly by invasive techniques such as taking blood samples (Snow, 1998). Tear collection may not be an equivalent substitute, but if certain immunological processes produce antibodies that leak from serum into the tear fluid, they could be monitored in the premature infant's closed eye tears. Thus the fluid collection becomes minimally invasive, less hazardous, and save both time and resources.

7.1.3 Implication for standard tear collection

The literature review showed that tear collection has been performed mainly in two ways. First the non-invasive tear collection with a glass capillary tube from adults and second the use of Schirmer paper that are invasive (van Haeringen and Glasius, 1977; Clinch et al., 1983; Stuchell et al., 1984; Craig and Blades, 1999; Yokoi and Komuro, 2004) but have been used also in infants and children (Marquardt and Wenz, 1980; Puderbach and Stolze, 1991; Spiegler and Mayer, 1993; Isenberg et al., 1998; Toker et al., 2002; Akar et al., 2004; Dogru et al., 2004; Rohatgi et al., 2005). Because of the possibility of adding serum proteins to the tear film, the latter method was rejected initially. Instead the alternative method was examined and compared with the glass capillary tube. Both collection methods are based upon the capillary attraction of fluid and in contrast to the capillary tube, the cellulose rod collected more tears when they were made available.

Therefore the cellulose rod is comparable to Schirmer paper. They are both absorbent materials and have a large surface area to maximise tear collection from the meniscus. The tear meniscus contains 75% to 90% of the aqueous and is correlated to the lacrimal secretion (Holly, 1985). Similar to collection with Schirmer strips, the duration of the collection was timed and the collected volume was only limited by the absorbance of the cellulose rod and availability of the tear fluid. This opens the door to further speculation whether the cellulose rod is measuring the tear fluid secretion rate. However, in contrast to the Schirmer paper and capillary tube, tear collection with the cellulose rod avoids the risk of injury to the ocular surface.

7.2 Review of the study design

7.2.1 Co-operation of infants and children

Co-operation is important for the recruitment and success of the tear collection from infant subjects, but it cannot be objectively evaluated. When planning this thesis, one of the concerns was that parents may reject the idea of an object close to the eyes of their newborns or the delicate premature infants. There was also no experience or reports on how participating infants would co-operate. However, the experience of patient recruitment was very positive. Parents who were interested in knowing about the study, reacted very positively when the cellulose rod was shown to them. Although parents were informed that, if they change their mind collection can be terminated at any time, none of the parents chose to intervene. This may be because the collection proved to be non-invasive and the babies were almost unaware of the collection procedure. Parents were also always helpful in securing the infant's head and arms.

Some premature infants were incubated and under observation. Therefore, tear collection was indicated only once infants were old enough to undergo the examination for retinopathy of prematurity. During the ophthalmologist's routine examination, it was observed that as a reaction to stress, the infant's heartbeat would change. The ophthalmologist would stop the examination until it normalised. The tear collection process was applied beforehand and such a reaction was never observed.

In contrast, tear collection time of the contact lens wearing children had to be shortened due to difficulties with co-operation. But, it was enough time to collect a

sufficient amount. Although children were co-operative for a shorter time, there were no reports of scratches to the conjunctiva.

7.2.2 Adult co-operation

When recruiting adults, one main concern was how many subjects will co-operate. Many subjects were students or staff and mostly used to similar trials. Despite this, some subjects showed nervousness when the cellulose rod or the capillary tube first approached the eye. In these subjects tear collection was delayed until they felt comfortable enough or it was repeated on another day. On the second tear collection appointment this nervousness was no longer present.

Results showed that the cellulose rod was able to collect both basal and stimulated reflex tears. In contrast, the capillary tube collected tears at a slower rate, even when stimulated. In adults, the cellulose rods can be used as an alternative to capillary tubes or both methods can be used depending on the patient's preference.

7.2.3 Improvement in collection succes rate

Arrangements were made to avoid variation in tear fluid, for example multiple tear collection from the same subject was made on the same day to avoid day to day variation and samples from different subjects were collected at the same time of each day to avoid fatigue. Inclusion criteria were tightened up by using a more stringent tear break-up time criteria and more detailed a questionnaire.

The percentage of collected samples from adult subjects was 85% (Chapter 4, Experiment 2) and increased to 96% in the last study with adults (Chapter 5). This reflects the improvement in skilled tear collection and the more stringent selection criteria for non-dry eye subjects in the last experiment.

7.3 Implications of this thesis on future studies

7.3.1 A new collection method and dry eye

This thesis did not apply the cellulose rod to other subject populations, but the results show that other applications are possible. Particularly subjects with dry eye would be of interest. Dry eye patients with deficient tear secretion are very challenging to any kind of tear collection method. Some investigators therefore recommend flushing the ocular surface before tear collection (Bjerrum and Prause, 1994), or of inducing reflex tearing non-invasively by yawning (Choy et al., 2003). This means that fluid is added to the tear film and a higher sample volume is collected. The tear content may be diluted but if all fluid on the ocular surface can be collected rapidly, the original tear fluid is still included. For the proposed methods the cellulose rod may be the ideal collection method since it can absorb tears quicker than the capillary tube and avoid loss by drainage.

In this thesis, the collection method aimed for non-stimulated tears. If reflex tearing occurred it was only in a few subjects. Future research addressed to dry eye needs to determine the maximum reflex tearing and dilution tolerable to sample assays, examine the use of larger cellulose rods and apply them to subjects with dry eye.

7.3.2 Safe tear collection and future study designs

This thesis found that cellulose rods are easy to handle and minimally invasive. They may be especially suitable for usage by subjects when not under supervision. These could be studies that involve repeated tear collection throughout the day or at awkward times and places. For example, they can be comfortably collected at home, even by children and parents and sent by special cooled delivery to the investigator. Cellulose rod tear collection by the subject involves less training and less hazard than collection by capillary tubes. Furthermore, tear samples from many more subjects can be collected in a short time.

7.3.3 Infant tear collection and future research

7.3.3.1 Impact on adult tear film studies

The protein analysis of infant tears showed a strong contribution of closed and open eye variables. These variables cause a similar variation in adult open and closed eye tears (Sitaramamma et al., 1998b; Fukuda and Wang, 2000; Sack et al., 2000). Mass spectrometric of adult open eye tears has shown more than 100 proteins in low concentration (Fung, Morris and Duncan, 2002). The range of proteins found comprised, amongst others, cytokines, growth factors and angiogenic factors, enzymes that can promote or inhibit inflammation and tumor growth. In adult closed eye tears, these enzymes are enriched which resembles a state of inflammation, such as in allergies or dry eye (Sack et al., 1992; Fukuda and Wang, 2000). Determining their exact concentrations and ratios between them may help to understand inflammation and find useful biomarkers (Sack et al., 2005). Studies on adults require that tears are collected immediately after waking up. These tears may be altered and

despite sensitive analysis important information may be lost. Instead the tears in infants can be studied more accurately. Studying closed eye tears in infants offers the advantage that infants do not wake up.

7.3.3.2 Impact of a new tear collection method on management of systemic diseases

A serious systemic disease with low life expectancy of about 30 years of age, that also affects the ocular surface and tear film, is cystic fibrosis (Davis, 2006). Cystic fibrosis is an inherited disease that affects the secretory epithelia of the lungs and also that of the eye. Screening for cystic fibrosis is carried out already in newborns (Davies, 2006). Dry eye in cystic fibrosis has been found to correlate with inflammatory cytokines (Mrugacz et al., 2006). Similar correlations have been found when chronic allergies were studied (Uchio et al., 2000). When investigating the tear film for significant levels of cytokines to be used as biomarkers in these diseases, included subjects are often young children (Kalayci et al., 1996; Leonardi et al., 2003). The cellulose rod being an ideal method to collect infant's and children's tears for these investigations, it may also be a useful tool for mass screening. In a future study, the inflammatory agents in the tear film of infants and children with systemic diseases can be studied to test its reliability as a sensitive tool.

7.3.3.3 Paediatric contact lens wearers

Paediatric contact lens wearers is an area of future research and, for the first time, they have been addressed in this thesis. Information, such as contact lens types, ocular surface problems and the effect of other treatments were collected and will improve

experimental designs. Tear collection and analysis, such as for tear ferning, have been used to forecast adult contact lens tolerance (Ravazzoni et al., 1998) and may have the clinical benefit of monitoring contact lens tolerance in children.

7.4 CONCLUSION

Until now research on infant tears has been motivated by questions that relate to the onset and maturation of secretion. The biochemistry of infant tears has been widely neglected. The work in this thesis developed a method of tear collection by the cellulose rod that was useful to subjects without head or eye movement control. Nevertheless, it did not look at disease states such as dry eye. The knowledge gained will help in understanding tear function and development of healthy tears. The development of a tear collection method for infants may also open the path to research on infants with abnormalities, such as cystic fibrosis or Down's syndrome. At the same time, the experience will create new ideas on how to design further studies on the biochemistry of infant tears.

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Appendix 1: Ethical approvals

Appendix 2: Information sheets, consent forms and questionnaires for recruitment

2.1. Adult consent form

2.2. McMonnies modified dry eye questionnaire

2.3. Parent information sheet and consent forms (English)

2.4. Parent information sheet and consent form (Welsh)

2.5. Recruitment poster for infants

2.6. Appendix 6: Recruitment poster (Welsh)

2.7. Parent information sheet and consent form for paediatric contact lens wearer

2.8. Information about tear collection in paediatric contact lens wearer

Department of Optometry and Vision Sciences

Subject Consent Form

An Investigation of the Effect of Tear Collection Techniques on Tear Composition

The pre-ocular tear-film has a number of important roles in the function of the healthy cornea. It provides a smooth optical surface for the ocular optical system, it provides nutrition to the cells of the anterior ocular surface, and it assists in the blink mechanism by providing a lubricant to the movement of the lids. Together with the blinking action the tears remove any foreign body in the eye.

In certain situations this normal functioning of the tear film breaks down. This can have pathological or environmental causes, and can produce subjective symptoms like redness and/or itchyness of the eye. Understanding the source of these symptoms and finding suitable management options has been difficult. Indeed for understanding the tear film we need further investigations of its normal composition.

To help us in this study, we will only need to take about 3 minutes of your time, in 2 sessions. We will collect tears from your eyes using a glass tube, and a cellulose rod. You might feel a slight sensation at your eye lid which is not painful or unpleasant. We will take tear samples from you and will find the best and easiest tear collection methods to improve our results.

If you have any questions to ask about the study, please ask me. My name is Marieh Esmaeelpour, and I am a PhD student. You can speak to me or to my supervisor, whose name is Dr. Paul J Murphy.

Declaration

I have read and understood the above information and have had the opportunity to ask questions before proceeding. I agree to participate in the project. I am aware that:-

a) This does not compromise my rights in law

b) I can withdraw from this experiment at any time and no sanctions will be taken against me for doing so. My withdrawal will not affect the standard of clinical care that I receive.

Signed ..... Date .....

School of Optometry and Vision Sciences

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

An Investigation of the Effect of Tear Collection Techniques

Please answer the following questions:

Name: _____

Age:
Male ○ Female ○
Do you take any medications? (If yes, please list the medications)

_____

Do you have any history of eye problems? (If yes, please give more details):

_____

Dry Eye

Do your eyes ever feel:

symptom	yes	no	Time of day	
sore			morning ○	evening ○
red			morning ○	evening ○
gritty			morning ○	evening ○
itchy			morning ○	evening ○
burning			morning ○	evening ○
light sensitive			morning ○	evening ○
tearing			morning ○	evening ○
other:			morning ○	evening ○

Are the symptoms made worse by:

	yes	no	Time of day	
computer work			morning ○	evening ○
air conditioner			morning ○	evening ○
heating			morning ○	evening ○
other			morning ○	evening ○

Do you have any dryness of the mouth/throat/chest?

	yes	no	Time of day	
mouth			morning ○	evening ○
throat			morning ○	evening ○
chest			morning ○	evening ○
other			morning ○	evening ○

Do you take any eye drops or other medication to relieve your symptoms? (If yes, please list the medications).

_____

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PRIFYSGOL CAERDYDD

Parent Information Sheet

Human Neonate Pre-Corneal Tear Films Physiology

You may have observed that a baby blinks very infrequently. This is because the tears in the eyes demonstrate a very high level of stability. But the tear-film undergoes a gradual change in its structure and composition during the first few months and years following birth, progressing towards a more 'adult' situation.
The tear-film has a number of important roles in the function of the healthy eye. It provides a smooth surface, it provides nutrition to the cells of the ocular surface, and together with the blinking action the tears remove any foreign body in the eye.
In certain situations this normal functioning of the tear-film breaks down. This can have pathological or environmental causes, and can produce redness and/or itchyness of the eye. Understanding the source of these symptoms and finding suitable management options has been difficult. Indeed for understanding the tear-film we need further investigations of its healthy composition.

To help us in this study, we will only need to take a little sample of the baby tears. We will collect tears with a soft rod while the head is held in a resting position. This method does not cause any discomfort and has been proven to be non-invasive. There might be a slight sensation at the eye lid which is not painful or unpleasant. We will take tear samples very quickly in only 2 minutes. We will also examine the medical record for gestational age, birth weight, and weight at last measurement.
A reimbursement of travel expenses will be made if your visit does not fit in with the normal medical schedule.
At the end of the study our results may improve care for people with dry eyes and infants that need to wear contact lenses.

My name is Marieh Esmaeelpour, and I am a PhD student. You can speak to me or to my supervisor, whose name is Dr. Paul J Murphy.
For questions, at any time, you can contact us on 029 2087 4703.

If you are willing to enrol your child in the study, please complete the following consent form.

Version 2 Reference number 04/WSE02/125 Date 8/11/2004

Parent consent form

Human Neonate Pre-Corneal Tear Films Physiology

I have read and understood the information provided (Patient Information Sheet Version 2 Reference number 04/WSE02/125 Date 8/11/2004) and have had the opportunity to ask questions. ☐

I am willing to enrol my child in the study ☐

I understand that I am free to withdraw my child at any stage, without affecting the standard of care that my child receives, without having to provide an explanation and without that decision affecting current or future medical care in any way ☐

I would like to receive information about the study results at the end ☐

Child's Name _____

Signed _____ Date _____

Your Name _____

Version 2 Reference number 04/WSE02/125 Date 8/11/2004

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

FFURFLEN WYBODAETH I'R RHIANT / GWARCHODWR

FFISIOLEG HAENAU DAGARU BLAEN-GORNBILENNOL

Efallai i chi sylwi mai pur anaml mae eich babi yn blincio. Y rheswm am hyn yw bod y dagrau mewn llygaid yn sefydlog iawn. Ond mae strwythur a chyfansoddiad yr haen ddagrau yn newid yn raddol dros y misoedd a'r blynyddoedd cyntaf yn dilyn genedigaeth, gan ddatblygu tuag at lefelau 'oedolyn'.

Mae'r haen ddagrau'n cyflawni nifer o swyddogaethau pwysig i sicrhau bod y llygad yn iach. Mae'n darparu arwyneb llyfn, a maeth i gelloedd arwyneb y llygad, ac ynghyd â blincio, mae'r dagrau yn cael gwared ar unrhyw ddarnau estron o'r llygad.

Mewn rhai sefyllfaoedd, ceir namau yn swyddogaethau arferol yr haen ddagrau. Gall hyn ddigwydd oherwydd rhesymau patholegol neu amgylcheddol, a gall beri i'r lygad gochi a chosi. Mae'n anodd deall tarddiad y symptomau yma a chael atebion i'w rheoli, felly er mwyn deall yr haen ddagrau mae angen i ni ymchwilio ymhellach i'w chyfansoddiad iach.

Er mwyn ein helpu gyda'r astudiaeth yma, fe fydd angen i ni gymryd sampl bach iawn o ddagrau'r babi. Byddwn yn casglu'r dagrau drwy ddefnyddio rhoden feddal, tra fod pen y babi yn cael ei ddal fel wrth orffwys. Nid yw'r dull yma yn peri unrhyw boen, ac mae wedi ei brofi i fod yn ddull anymwthiol. Mae'n bosib y bydd y babi yn teimlo'r rhoden ar yr amrannau, ond ni fydd hyn yn boenus nac yn annifyr. Byddwn yn casglu'r samplau yn gyflym iawn, bydd popeth drosodd mewn 2 funud. Byddwn hefyd yn archwilio'r cofnod meddygol ar gyfer y cyfnod cario, pwysau ar enedigaeth a phwysau y tro diwethaf y cafodd ei bwyso.

Byddwch yn derbyn costau teithio os nad oedd eich ymweliad yn cyd-fynd â'r amserlen feddygol arferol.

Ar ddiwedd yr astudiaeth, fe fyddwn yn defnyddio ein canlyniadau i wella'r gofal a ddarpeir i bobl sydd yn dioddef o lygaid sych a phlant ifanc sy'n gorfod gwisgo lensys-cyffwrdd.

Fy enw i yw Marieh Esmaeelpour, ac 'rwy'n astudio ar gyfer fy noethuriaeth. Gallwch siarad a mi neu gyda'm goruchwyliwr, Dr. Paul Murphy.
Oes oes gennych gwestiynau, ffoniwch ni ar 029 2087 4703.

Os ydych yn barod i gofrestru eich plentyn yn yr astudiaeth, llenwch y ffurflen ganiatâd ganlynol.

Fersiwn 2 Cyfeirnod 04/WSE02/125 8/11/2004

Ffurflen Ganiatâd Rhiant

FFISIOLEG HAEN DDAGRAU CYN-GORNBILENNOL PLANT NEWYDD-ANEDIG

Rwyf wedi darllen a deall y wybodaeth a gefais (FFURFLEN WYBODAETH I'R RHIANT / GWARCHODWR Fersiwn 2 Cyfeirnod 04/WSE02/125 8/11/2004), ac wedi cael cyfle i ofyn cwestiynau ☐

Hoffwn gofrestru fy mhlentyn i gymryd rhan yn yr astudiaeth ☐

Deallaf fod gennyf yr hawl i dynnu fy mhlentyn o'r astudiaeth ar unrhyw adeg, heb i hyn effeithio ar safon y gofal a gaiff fy mhlentyn, heb orfod rhoi esboniad a heb fod y penderfyniad hwnnw'n effeithio ar ofal meddygol mewn unrhyw ffordd yn awr nac yn y dyfodol ☐

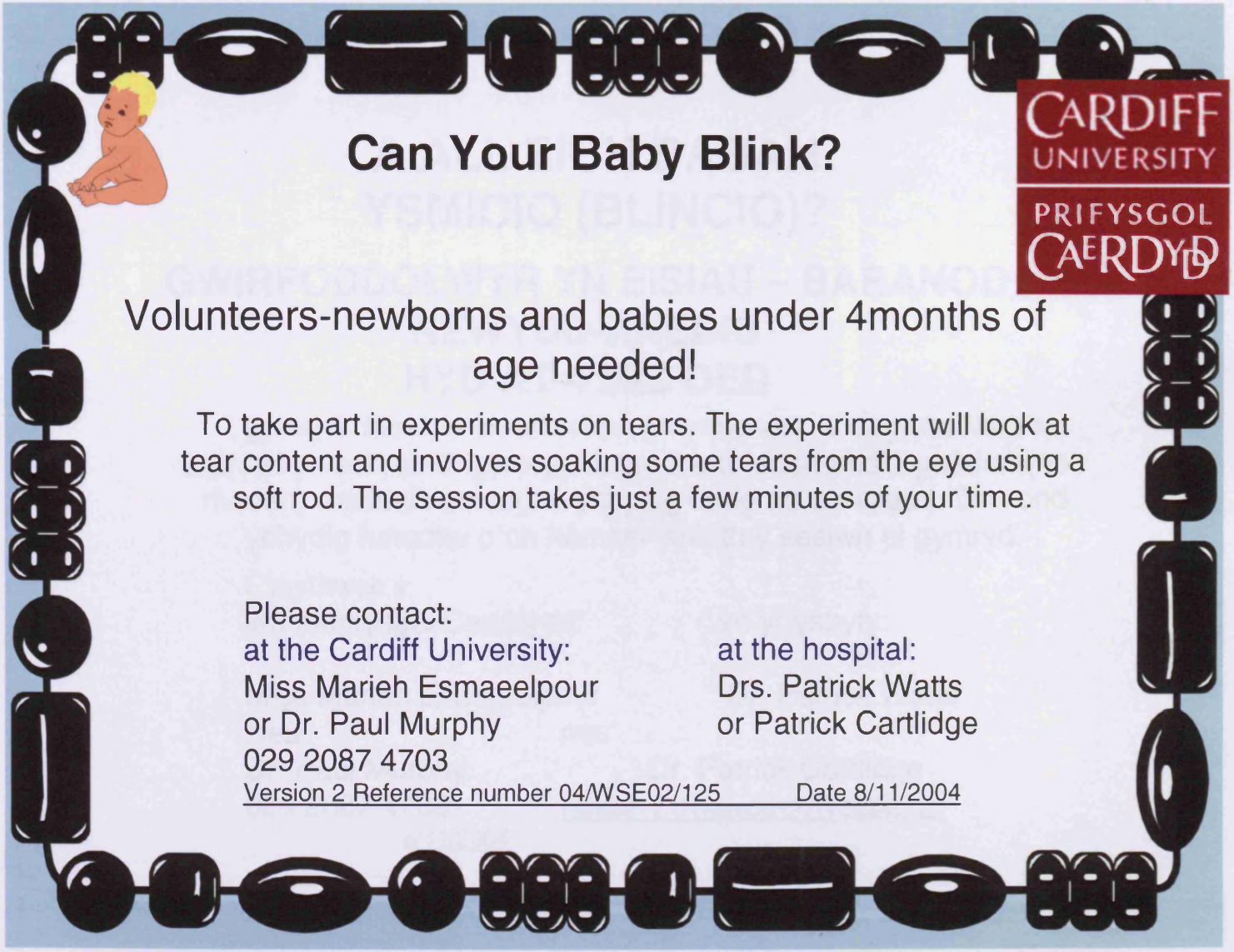
Hoffwn dderbyn gwybodaeth am ganlyniadau'r astudiaeth ar y diwedd ☐

Enw'r plentyn _____

Arwyddwyd _____ Dyddiad _____

Eich enw chi _____

Fersiwn 2 Cyfeirnod 04/WSE02/125 8/11/2004


Can Your Baby Blink?
CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD
Volunteers-newborns and babies under 4months of age needed!
To take part in experiments on tears. The experiment will look at tear content and involves soaking some tears from the eye using a soft rod. The session takes just a few minutes of your time.
Please contact:
at the Cardiff University:
Miss Marieh Esmaeelpour
or Dr. Paul Murphy
029 2087 4703
at the hospital:
Drs. Patrick Watts
or Patrick Cartlidge
Version 2 Reference number 04/WSE02/125 Date 8/11/2004

A ALL EICH BABAN YSMICIO (BLINCIO)?

GWIRFODDOLWYR YN EISIAU – BABANOD NEWYDD-ANEDIG HYD AT 4 MIS OED

I gymryd rhan mewn arbrofion yn ymwneud a dagrau. Mae'r arbrawf yn edrych ar gynwys dagrau wrth ddefnyddio gwialen (neu rhoden) meddal i amsugno ychydig ddagrau o'r llygad. Dim ond ychydig funudau o'ch hamser wneith y sesiwn ei gymryd.

Cysylltwch a:

ym mhrifysgol Caerdydd:

Miss Marieh Esmaeelpour

Neu

Dr. Paul Murphy

029 2087 4703

yn yr ysbyty:

Dr. Patrick Watts

neu

Dr. Patrick Cartlidge

Fersiwn 2 Cyfeirnod 04/WSE02/125

8/11/2004

Parent consent form

Human Neonate Pre-Corneal Tear Films Physiology

I have read and understood the information provided (Patient Information Sheet Version 2 Reference number 04/WSE02/125 8/11/2004) and have had the opportunity to ask questions. ☐

I am willing to enrol my child in the study ☐

I understand that I am free to withdraw my child at any stage, without affecting the standard of care that my child receives, without having to provide an explanation and without that decision affecting current or future medical care in any way ☐

I would like to receive information about the study results at the end ☐

Child's Name _____

Signed _____ Date _____

Your Name _____

Version 2 Reference number 04/WSE02/125 Date 8/11/200

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

Parent Information Sheet

Human Neonate Pre-Corneal Tear Films Physiology

You may have observed that a baby blinks very infrequently. This is because the tears in the eyes demonstrate a very high level of stability. But the tear-film undergoes a gradual change in its structure and composition during the first few months and years following birth, progressing towards a more 'adult' situation.
The tear-film has a number of important roles in the function of the healthy eye. It provides a smooth surface, it provides nutrition to the cells of the ocular surface, and together with the blinking action the tears remove any foreign body in the eye.
In certain situations this normal functioning of the tear-film breaks down. This can have pathological or environmental causes, and can produce redness and/or itchyness of the eye. Understanding the source of these symptoms and finding suitable management options has been difficult. Indeed for understanding the tear-film we need further investigations of its healthy composition.

To help us in this study, we will only need to take a little sample of the baby tears. We will collect tears with a soft rod while the head is held in a resting position. This method does not cause any discomfort and has been proven to be non-invasive. There might be a slight sensation at the eye lid which is not painful or unpleasant. We will take tear samples very quickly in only 2 minutes.
The tear samples will be collected during the course of contact lens review assessments. The collection of tear samples on these occasions will not involve unnecessary removal, insertion or handling of the extended wear contact lens. Appropriate samples may be collected in the theatre when your child is asleep if ophthalmic examinations have been requested under anaesthesia. We will also examine the medical record for gestational age, birth weight, and weight at last measurement.
A reimbursement of travel expenses will be made if your visit does not fit in with the normal medical schedule.
At the end of the study our results may improve care for people with dry eyes and infants that need to wear contact lenses.

My name is Marieh Esmaeelpour, and I am a PhD student. You can speak to me or to my supervisor, whose name is Dr. Paul J Murphy.
For questions, at any time , you can contact us on 029 2087 4703.
If you are willing to enrol your child in the study, please complete the following consent form.

An Investigation of the neonate tear film

Name: _____
Code:
Date of birth:
Male ○ Female ○

If infant, up to 4 months:
Gestational age:
Birth weight:
Actual weight:

Contact lenses

Type of contact lens
Soft ○ RGP ○ Silicone ○ Silcone Hydrogel ○
First fitting :
wearing time: day and night up to 28days ○
day and night extended wear up to 7days ○
daily14hs ○
daily 6-8hs ○

What is the previous contact lens wearing history?

_____

History of contact lens problems?

_____

History of eye surgery? When?

_____

History of eye infections, inflammations?

_____

Do you take any eye drops or other medications? (If yes, please list the medications).

_____

Other systemic medications?

_____

What contact lens cleaning and soaking solutions and systems are used?

_____

How long has the current contact lens been in situ?

_____

Tear collection

Time of the day:

Duration of tear collection (if not 2min):

R eye ○ L eye ○

Used anaesthetics before tear collection?
Yes ○ No ○
If yes, which?

While tears were collected:
tearing ○ asleep ○ crying ○
eye was hold open? Yes ○ No ○
with CL? Yes ○ No ○

Appendix 3: Buffer solutions for Western Blotting

Buffers used for Western Blotting were made up:

- transfer buffer, for 100ml:

10ml of 10xTris/Caps (Bio-Rad Laboratories Ltd., UK)

15ml methanol (Fisher Scientific, UK)

and 75ml distilled water

- blocking buffer

10% skimmed milk powder (Santa Cruz Biotechnology, Germany)

50mM sodium fluoride (NaF) (Sigma-Aldrich Co, UK)

0.05% Tween20 (Sigma-Aldrich Co, UK)

- washing buffer

5% skimmed milk powder (Santa Cruz Biotechnology, Germany)

50mM sodium fluoride (NaF) (Sigma-Aldrich Co, UK)

0.05% Tween20 (Sigma-Aldrich Co, UK)

- stripping buffer

100mM -2 mercaptoethanol

62.5 mM Tris/HCL (Sigma-Aldrich Co, UK)

2% SDS (Sigma-Aldrich Co, UK)

Appendix 4: Results

Chapter 2
results show volumes in µl

Polyester rod					
Solution	Duration (min)	3	10	20	30
	Force: **15300xg**				
Opti-Tears™		7.2	7.8	6.3	7
		8.1	8.5	8	7.6
		8.8	8.3	8.8	8.5
		8.6	8.2	8.6	8.3
		8.8	8.5	8.3	8.5
Hylo-Prompt™		8.8	9	8.2	7.3
		8.7	8.9	8.5	8
		8.6	8.5	7.7	7.8
		9	8.6	8.6	8.5
		8.7	8.8	8.3	8
Saline		9.5	9.5	9.2	9.2
		9.3	9.4	9.1	9.2
		9.2	9.5	9.2	9.3
		9.5	9.4	9.1	9.4
		9.2	9.5	9.1	9
	Force: **5700xg**				
Opti-Tears™			7.8		
			8.6		
			8.9		
			8.6		
			8.3		
Hylo-Prompt™			8.6		
			8.9		
			8.6		
			8.7		
			8.8		
Saline			9		
			9.2		
			9		
			9.1		
	Force: **700xg**				
			9		
Opti-Tears™			7.7		
			8.5		
			8.4		

			8.4		
			7.8		
Hylo-Prompt™			8.1		
			8.0		
			8.5		
			8.1		
			8.4		
Saline			8.6		
			8.8		
			8.8		
			8.9		
			8.7		
Cellulose rod					
Solution	Duration (min)	3	10	20	30
	Force: 15300xg				
Opti-Tears™		6.0	7.7	7.0	7.2
		8.0	6.9	7.6	7.9
		8.0	7.8	7.6	7.5
		7.8	8.1	8.1	7.7
		6.8	7.9	7.7	8.1
Hylo-Prompt™		7.5	8.3	6.2	6.6
		8.0	7.8	7.4	7.7
		8.1	7.9	7.8	7.7
		7.9	8.0	7.6	6.7
		7.3	7.9	8.4	8.0
Saline		8.1	8.3	8.1	8.0
		8.0	7.3	7.9	8.1
		8.0	8.0	7.8	8.1
		7.8	7.9	8.1	8.2
		8.1	7.8	8.1	8.2
	Force: 5700xg				
Opti-Tears™			7.8		
			6.5		
			7.5		
			7.6		
			7.2		
Hylo-Prompt™			7.2		
			6.9		
			6.9		
			7.1		
			6.7		
Saline			7.6		
			7.5		

			7.5		
			7.5		
	Force: **700xg**				
Opti-Tears™			7.8		
			6.8		
			6.8		
			6.6		
			6.8		
Hylo-Prompt™			5.7		
			5.3		
			6.2		
			6.2		
			6.6		
Saline			7.1		
			7.5		
			7.1		
			7.2		
			6.8		
Cellulose sponge					
Solution	Duration (min)	3	10	20	30
	Force: 15300xg				
Opti-Tears™		7.3	7.3	8.0	8.0
		7.8	7.5	7.5	7.5
		8.0	7.8	7.7	8.5
		7.6	7.5	7.2	7.5
		7.3	7.6	7.7	7.3
Hylo-Prompt™		7.2	7.2	7.3	7.5
		7.3	7.0	7.5	7.3
		7.4	7.6	7.6	7.5
		7.5	7.1	7.5	7.2
		7.3	7.3	7.5	7.3
Saline		7.3	8.0	6.9	7.8
		7.1	7.0	7.8	7.6
		7.5	7.9	7.5	7.7
		7.6	7.7	7.3	7.8
		7.4	7.7	7.3	7.6
	Force: **5700xg**				
Opti-Tears™			7.2		
			7.3		
			7.4		
			7.4		
			7.4		
Hylo-Prompt™			7.1		

			7.3		
			7.3		
			7.3		
			7.6		
Saline			8.0		
			7.8		
			7.7		
			7.5		
	Force: 700xg		7.6		
Opti-Tears™			6.5		
			7.5		
			7.1		
			7.5		
			7.0		
Hylo-Prompt™			7.3		
			7.4		
			7.1		
			7.0		
			6.9		
Saline			7.0		
			7.0		
			7.0		
			6.9		
			7.0		
Schirmer paper					
Solution					
	Duration (min)	3	10	20	30
	Force: 15300xg				
Opti-Tears™		6.2	6.1	4.5	6.1
		6.5	6.3	6.9	6.1
		6.1	6.4	5.7	6.2
		5.5	6.4	5.5	6.3
		5.5	6.3	4.5	6.5
Hylo-Prompt™		6.1	6.1	5.5	6.8
		6.0	6.9	5.8	6.2
		5.8	6.2	6.2	6.2
		6.0	6.5	6.5	6.0
		6.4	6.8	6.4	6.5
Saline		6.7	6.5	6.5	7.4
		6.5	7.3	6.7	6.7
		6.0	6.7	6.0	7.2
		6.3	6.5	6.6	7.2
		6.4	7.0	6.9	7.1
	Force: 5700xg				
Opti-			5.0		

Tears™			5.6		
			5.5		
			6.0		
			4.8		
Hylo-Prompt™			5.7		
			6.0		
			5.4		
			6.0		
			6.3		
Saline			7.0		
			6.7		
			7.1		
			6.8		
	Force: 700xg		7.2		
Opti-Tears™			4.5		
			4.0		
			3.2		
			3.2		
			3.8		
Hylo-Prompt™			4.8		
			3.9		
			4.5		
			3.7		
			3.6		
Saline			5.0		
			4.5		
			4.4		
			5.0		
			5.5		
PVA spears					
Solution	Duration (min)	3	10	20	30
	Force: 15300xg				
Opti-Tears™		4.8	3.2	3.4	3.9
		4.7	3.9	3.8	4.0
		4.0	4.0	3.7	3.7
		4.6	4.2	4.5	4.8
		5.2	4.1	3.3	3.6
Hylo-Prompt™		5.6	4.7	4.8	3.4
		5.4	4.3	5.5	4.2
		4.0	4.7	4.7	4.0
		4.7	4.4	6.0	4.0
		6.5	4.3	4.8	4.7
Saline		4.3	3.9	3.3	3.5
		2.9	3.7	3.7	2.4

		3.0	3.1	3.4	4.6
		3.0	3.3	3.8	3.6
		2.9	3.6	3.4	3.6
	Force: 5700xg				
Opti-Tears™			2.3		
			3.2		
			2.6		
			3.7		
			5.3		
Hylo-Prompt™			4.6		
			5.0		
			4.2		
			5.0		
			3.1		
Saline			3.6		
			3.1		
			3.1		
			3.0		
	Force: 700xg				
Opti-Tears™			3.0		
			2.5		
			4.0		
			3.0		
			2.5		
Hylo-Prompt™			2.5		
			3.5		
			3.2		
			3.3		
			4.0		
Saline			2.3		
			2.0		
			2.7		
			2.7		
			2.5		
cellulose spears					
Solution					
	Duration (min)	3	10	20	30
	Force: 15300xg				
Opti-Tears™		1.1	1.4	.7	1.6
		.0	.2	1.0	1.0
		1.4	1.5	1.1	.6
		.3	.4	.3	.9
		.8	.0	.7	1.5
Hylo-Prompt™		1.5	2.2	1.1	.3

		.6	1.2	1.6	.8
		.9	1.5	1.5	.9
		.3	.7	1.1	.5
		.8	.0	.5	1.5
Saline		.1	1.0	.6	.0
		.8	.3	.5	.5
		.3	.4	.7	.0
		.5	1.2	1.1	.8
		2.0	.4	.2	.0
	Force: **5700xg**				
Opti-Tears™			1.0		
			.8		
			.9		
			.3		
			.4		
Hylo-Prompt™			1.2		
			.3		
			.3		
			.2		
			.0		
Saline			.0		
			.0		
			.0		
			.0		
	Force: **700xg**				
Opti-Tears™			.0		
			.0		
			.0		
			.1		
			.2		
Hylo-Prompt™			.3		
			.3		
			.2		
			.0		
			.0		
Saline			.0		
			.0		
			.0		
			.0		
			.0		

Remaining factors:

Polyester rod			
	stored	sterile	room-temperature
Opti-Tears™	8.3	9	8.3
	9.1	8.4	7.7
	9.2	8.9	8.2
	9.5	9.1	8.1
	9	8.2	7.8
Hylo-Prompt™	9	8.7	8.1
	9.3	8.6	8.3
	9.5	8	8.5
	9.5	8.5	8.4
	9.3	8.7	8.5
Saline	9.8	8.7	9
	9.2	9	9.1
	9.3	8.9	9.1
	9.2	8.8	9.2
	9.5	8.6	9

Cellulose sponge			
	stored	sterile	room-temperature
Opti-Tears™	8	7.5	7.6
	7.5	7.7	7.3
	7.5	7.5	7.9
	8.5	7.5	7.6
	7.5	7.8	7.5
Hylo-Prompt™	7.5	7.9	7.7
	7.7	8	7.3
	7.7	8.4	7.3
	7.6	7.6	7.6
	7.7	7.5	7.3
Saline	7.8	7.5	8
	7.5	7.2	7.1
	7.9	7.3	7.3
	7.6	7.3	7.2
	7.7	7.6	7.3

Chapter 4

The *in vitro* study

Results for the cellulose rod

fresh samples			stored samples		
Bovine serum albumin concentration (μg/μl)					
2.58	2.92	2.48	1.68	2.28	2.18
2.04	2.64	1.66	1.64	2.22	1.84
2.22	2.48	2.74	2.16	2.24	2.5
2.2	2.78	2.9	1.88	2.08	2.7
2.3	2.48	2.08	2.04	2.84	2.84
2.3	2.5	2.5	1.5	2.84	2.7
Extracted volume (μl)					
4	3.7	3.1	4	3.5	3.6
4.2	3.9	4.1	4.2	3.7	3.2
4.3	4.2	3.8	3.7	4	4
4.3	4	4	4	3.7	3.8
3.9	3.6	4	3.7	3.8	3
3.9	3.3	4.3	3.7	3.2	3.6

Results for the capillary tube

fresh samples			stored samples		
Bovine serum albumin concentration (μg/μl)					
2.08	1.46	2	1.3	1.28	1.86
2.16	1.92	1.6	1.84	1.36	2.2
1.9	1.86	1.7	1.6	1.44	1.64
1.76	1.84	1.7	1.2	1.2	1.98
1.96	1.78	1.62	1.64	1.72	1.82
1.8	1.58	1.86	1.97	1.4	2.05
Extracted volume (μl)					
4.9	5	4.7	1.5	5	5
5	5	4.7	4.5	4.7	5
4.8	4.8	5	4	5	4.9
5	5	5	2.8	5	4.8
5	4.6	4.5	4.6	5	5
5	5	5	3.6	5	4.8

The *in vivo* study
Results from seven subjects

Cellulose rod

fresh samples			stored samples		
Tear protein concentration (μg/μl)					
8	15	18.8	15.3	10.4	13.3
9.6	16	15.9	16.5	13.5	11.5
8.8	16.8	14.3	11.5	9	11.5
15.3	10.2	8.8	9.7	15.3	9.4
12.2		15.2	13.3	11.1	10.2
16.2	16.7	15	12	11.8	15.6
9.9		16.8			14.9
Extracted tear volume (μl)					
8	3	5.5	7.5	9	3
15	11	0.5	1	0.8	1
20	2	7	15	7	20
13	12.5	25	8.5	2	4.5
0.5	0	1	1.7	1.2	1
0.8	2.5	1.5	7.5	3.5	0.7
0.8	0	2.5	0	0	2

Capillary tube

fresh samples			stored samples		
Tear protein concentration (μg/μl)					
7.6	15.5	11.6	7.4	12.4	14.6
12	14.4	12.2	10.6	12.7	15.6
3.8	8.3	16.5	7	11.4	13.6
8.2	15.3	14.2	14.7	9.4	13.7
9.65	10.5	9.7	8.15	14.1	9.1
7.8	8.3	13.6	8.7	7.8	7.65
7.8	16.8	16	7.4	10.6	14.7
Extracted tear volume (μl)					
2.3	3.5	3	2	3	5
6	1.5	2.3	4	3	2
1.7	2	1	2.1	2	2.5
3.3	1.4	1.5	1.7	1.3	5
3.5	4	2.5	3.1	2	2.5
2.3	3.3	3.3	6	3.1	2.7
2.5	1.5	1.5	3	1.5	1.9

Tear stimulation study
Results from 6 subjects

non-stimulated samples		stimulated samples	
capillary tube	cellulose rod	capillary tube	cellulose rod
Tear protein concentration (μg/μl)			
	14.8	9.2	11.9
12.4	13.8	11.8	11.8
18.2	20	8.6	8.9
9.6	10.6	11	7.6
15.6	9.8	15.3	9.2
13.7	9.4	10	8.8
Extracted tear volume (μl)			
0.3	6	3.5	4
1.2	2.8	1	3.8
0.5	1	3.5	50
1.2	7	4	25
3	22	1	48
0.6	10	3	24

Results for technique invasiveness study (CR=cellulose rod, CT=capillary tube)

subjects	volume CR (µl)	volume CT (µl)	protein conc. CR (µg/µl)	protein conc. CT (µg/µl)	SA ratio CR	SA ratio CT
1	5	2.3	12.5	14.2	0.35112	0.234132
2	0.2	1	.	.	.	.
3	0.4	1	.	.	0.354095	0.284231
4	0	1	.	.	.	.
5	3.3	0.9		.	0.663949	0.261663
6	0.5	1.5	.		0.654926	0.350036
7	0	0.5	.	.	.	.
8	26.7	1.5	5.6	7.1	0.171481	0.150567
9	2.5	1.7	8.8	12.3	0.408041	0.207244
10	0	0.5	.	.	.	.
11	8.3	3	9.7	9.5	0.249144	0.272153
12	0.8	2	.		0.619781	0.401331
13	2.3	1.7		.	0.412635	0.346212
14	0.6	2	1.7	7.2	0.592816	0.700505
15	.	0.4	.	.	.	
16	1.8	1.7	9.4	3.5	0.491055	0.29788
17	0.4	1.8	.		0.590836	0.31694
18	20	2	6.4	4.4	0.107918	0.081451
19	2.1	2.2	12.2	1.7	0.415073	0.100437
20	2.7	1.3	16.1	7.9		.
21	0.8	1.5	.		0.805478	0.362325
22	22	2	10.2	3.3	0.209791	0.221595
23	4.5	1.8	7.6	6.8	0.18734	0.27816
24	8	2	14.9	13.1	0.506813	0.357682
25	0.4	1.7	.		0.512617	0.336596
26	3.2	0.6		.	0.326995	0.32747
27	1.7	1.5	.	.	0.427897	0.307301

subject	volume CR (µl)	volume CT (µl)	IgA conc. CR (µg/µl)	IgA conc. CT (µg/µl)	Lactoferrin CR (µg/µl)	Lactoferrin CT (µg/µl)	Lysozyme CR (µg/µl)	Lysozyme CT (µg/µl)
1	4	2	3.14	3.65	4.8	3.49	5.3	4.28
2	2	2	3.15	1.3	2.55	2.72	4.15	3.87
3	9.5	2	1.84	1.91	2.36	1.58	2.04	1.67
4	11	2.3	0.49	0.99	2.23	1.19	1.48	1.27
5	0	2						
6	2.5	2	2.58	2.1	1.94	2.5	1.2	1.7
7	3	3	1.52	1.99	3.05	3.3	2.12	2.2
8	6	1.5	1.68		1.35		2.2	
9	4	2	2.18	2.3	1.99	2.12	2.63	3.6
10	4	2.5	2.85	2.04	2.74	2.1	5.97	5.5
11	30	2.5	1.25	0.64	1.14	0.65	1.88	1.6
12	18	1.5	2.2	1.12	1.87	1.5	0.6	1.6
13	10	2	1.33	1.35	1.4	1.22	1.49	1.6
14	20	3	0.94	1.9	1.64	2.42	1.45	2.41
15	0.4	1.5						
16	0	0.8						

Results for the major protein concentration study (CR=cellulose rod, CT=capillary tube)

Chapter 5

Results are given in the order of collection
P1
(BW=birth weight, GA=gestational age, PCA=post-conceptional age)

subject	gender	BW	actual weight	GA	PCA	alertness
1	male	0.64	1.85	24	35	crying
2	female	2.11	2.9484	31.3	39	crying
3	male	1.625	2.145	31.9	36.2	crying
4	male	1.13	1.75	34	38	crying
5	female	0.72	1.45	29	37	closed eye
6	female	1.24	2.07	30	36	closed eye
7	female	1.32	1.91	30	36	closed eye
8	female	1.195	2.34	30.4	34.71	closed eye
9	male	1.08	1.705	30.6	35	closed eye
10	male	1.98	2.75	30.6	36.57	closed eye
11	male	1.36	2.15	31.4	37.42	closed eye
12	female	1.16	1.86	29.9	36.86	closed eye
13	female	1.535	2	31.3	36.2	closed eye
14	male	1.775	1.73	30.7	32.71	closed eye
15	female	2.2	2.31	35.7	37.57	closed eye
16	male	1.984	1.984	34	34.14	closed eye
17	male	0.905	1.815	28.9	35.86	closed eye
18	male	0.53	1.01	27.9	34	closed eye
19	female	0.69	1.26	27.9	34	closed eye
20	female	0.78	1.96	28.7	37	closed eye
21	male	0.64	0.89	27	35	closed eye
22	male	1.12	1.575	35	38	open eye
23	female	0.9	1.68	30	35.71	open eye
24	male	0.84	1.42	28.9	34.86	open eye
25	female	0.73	1.35	30.9	38	open eye
26	male	1.705	2.14	31.4	35.15	open eye
27	male	1.13	2.75	29	35	open eye
28	female	0.65	0.83	24.3	30	open eye
29	male	1	1.61	26	34	open eye
30	female	0.985	2.85	27	38	open eye

subject	volume (µl)	protein conc. (µg/µl)	IgA (µg/µl)	lactoferrin (µg/µl)	lysozyme (µg/µl)
1	6	12.8	1.093	0.824	5.164
2	15	8.4	1.096	1.812	1.05
3	3.5	.	0.927	1.309	1.251
4	0.1	.	.	.	.
5	0.8	9.25	1.714	2.184	.
6	3	17.25	1.6	3.29	7.76
7	3.5	20	3.517	4.634	12.146
8	0.4	.	.	.	.
9	4	.	1.48	2.186	1.5
10	0.5	.	1.669	3.633	6.54
11	0.5	.	2.297	3.043	8.55
12	4	9.9	0.907	1.832	3.913
13	0.3	.	.	.	.
14	3.5	9.4	1.744	2.098	4.042
15	1.5	16.4	1.181	2.254	3.504
16	1.2	.	0.671	1.432	3.078
17	0.4	.	.	.	.
18	1	.	0.447	0.41	0.471
19	2	18.2	0.865	1.599	3.302
20	2.3	12.3	0.89	0.888	3.479
21	0.2	.	.	.	.
22	0.5	.	.	.	.
23	3	16	1.329	1.584	3.755
24	0.2	.	0.648	0.994	0.67
25	7	7.4	.	.	.
26	0.5	.	0.698	1.416	3.375
27	1.5	9.7	1.225	1.804	2.418
28	3.2	.	0.406	0.945	0.942
29	1.7	13.5	0.681	0.94	1.229
30	2.7	5.9	0.577	1.264	1.49

P2

subject	gender	BW	actual weight	GA	PCA	alertness
1	female	1.24	2.38	30	37.57	crying
2	female	1.32	2.235	30	37.57	crying
3	female	0.72	1.7	29	39	closed eye
4	female	1.16	2.11	29.9	38.86	closed eye
5	male	1.775	2.5	30.7	37	closed eye
6	male	1.984	2.6	34	40.27	closed eye
7	female	0.65	1.39	24.3	34	closed eye
8	female	0.69	1.5	27.9	36	closed eye
9	female	0.9	2.03	30	37.71	open eye
10	male	0.64	1.95	24	37.27	open eye
11	male	0.69	1.33	27.9	36	open eye
12	male	1	1.87	26	35	open eye

subject	volume (µl)	protein conc. (µg/µl)	IgA (µg/µl)	lactoferrin (µg/µl)	lysozyme (µg/µl)
1	13	.	1.22	3.026	4.22
2	17	.	0.695	2.647	2.41
3	0.5	.	1.99	2.36	4.03
4	2	17.4	0.919	1.636	2.66
5	3.5	9.4	.	.	.
6	4.7	18.2	4.785	4.255	6.91
7	4.5	.	0.401	0.49	0.368
8	3.5	16.3	0.504	1.271	2.143
9	2.7	8.6	1.24	2.005	2.203
10	1	.	1.478	4.245	9.935
11	2	19.5	0.401	1.228	2.794
12	6.8	14.5	0.836	1.138	0.401

subject	SA (grade)	IgM presence	lipocalin presence	number of bands	fern (grades)
1	2	present	present	7	.
2	3	present	present	8	.
3	3	.	.	.	.
4	3	present	present	11	.
5	.	.	.	.	.
6	3	present	present	10	3
7	2	present	none	5	2
8	3	present	none	11	2
9	2	present	present	7	.
10	2	none	present	5	.
11	3	present	none	13	2
12	3		present	7	2

F1

subject	gender	BW	actual weight	GA	PCA	alertness
1	male	3.59	3.59	40	40.14	crying
2	male	3.35	3.35	37.3	37.42	crying
3	female	3.59	3.59	39.6	39.71	crying
4	female	2.64	2.64	39	39.14	crying
5	male	2.63	2.63	36	36.14	crying
6	male	3.35	3.35	39.6	39.71	closed eye
7	male	3.89	3.89	39	39.14	closed eye
8	male	3.77	3.77	42	42.14	closed eye
9	male	3.41	3.41	41.3	41.28	closed eye
10	male	3.53	3.53	40.1	40.27	closed eye
11	male	3.11	3.11	40.7	40.85	closed eye
12	male	3.36	3.36	40.4	40.57	closed eye
13	female	3.11	3.11	39.6	39.85	closed eye
14	female	3.11	3.11	39.4	39.57	closed eye
15	female	3.55	3.55	38.6	38.71	closed eye
16	female	4.2	4.2	38.7	38.85	closed eye
17	female	3.06	3.06	40.1	40.28	closed eye
18	male	3.52	3.52	37.8	38	closed eye
19	female	3.49	3.49	40.9	41	closed eye
20	female	3.22	3.22	39.9	40	closed eye
21	male	3.32	3.32	41	41.14	closed eye
22	female	3.06	3.06	40.9	41	closed eye
23	female	3.32	3.32	38.6	38.71	closed eye
24	female	2.79	2.79	39.4	39.57	closed eye
25	male	2.93	2.93	39.1	39.28	closed eye
26	female	2.75	2.75	39.6	39.71	closed eye
27	male	3.22	3.22	39	39.14	open eye
28	male	3.97	3.97	39.7	40	open eye
29	male	3.53	3.53	38	38.28	open eye
30	female	3.94	3.94	41.7	41.85	open eye
31	female	3.94	3.94	41.7	42	open eye
32	female	2.63	2.63	39.3	39.42	open eye
33	male	5.05	5.05	39.6	39.6	open eye
34	female	2.4	2.4	40	40.14	open eye
35	male	4.337	4.337	38.6	38.71	open eye
36	male	4.08	4.08	41.9	42	open eye
37	male	3.2	3.2	39.3	39.42	open eye
38	female	3.63	3.63	40.7	40.85	open eye
39	male	3.82	3.82	41	41.14	open eye
40	female	3.27	3.27	39.6	39.71	open eye

subject	volume (µl)	protein conc. (µg/µl)	IgA (µg/µl)	Lactoferrin (µg/µl)	lysozyme (µg/µl)
1	8	7.5	0.414	1.012	0.66
2	2	9	0.53	0.725	0.838
3	0.1	.	.	.	.
4	0.2	.	.	.	.
5	0	.	.	.	.
6	1	.	0.348	1.652	3.109
7	0.3	.	.	.	.
8	0	.	.	.	.
9	0.5	.	0.9	2.262	6.2
10	2.2	22	0.872	2.634	3.195
11	0	.	.	.	.
12	0	.	.	.	.
13	0	.	.	.	.
14	0.05	.	.	.	.
15	1.2	.	0.641	1.793	1.28
16	1	.	0.698	1.102	1.125
17	0.2	.	.	.	.
18	0	.	.	.	.
19	1.2	.	0.657	1.431	3.85
20	2.7	.	0.436	0.958	2.441
21	3.3	.	0.57	1.101	3.209
22	0	.	.	.	.
23	3.5	7.2	0.288	0.602	0.627
24	1	.	0.747	0.907	3.638
25	0.09	.	.	.	.
26	0.2	.	.	.	.
27	2	12.2	0.322	0.86	3.07
28	0	.	.	.	.
29	3.5	14.2	1.034	2.594	3.14
30	0.5	.	0.692	1.211	1.285
31	1	.	0.428	1.168	3.715
32	6	11.5	0.117	1.189	2.021
33	2	.	1.302	1.741	2.275
34	0.1	.	.	.	.
35	0.1	.	.	.	.
36	1	.	0.919	1.513	3.342
37	7	4	0.403	0.628	1.716
38	0.5	.	0.413	0.71	2.091
39	0	.	.	.	.
40	1.2	.	0.521	0.989	0.918

subject	SA (grade)	IgM presence	Lipocalin presence	number of bands	fern (grades)
1	2	present	none	5	2
2	3	present	none	13	1
3	.	.	.	.	1
4	.	.	.	.	.
5	.	.	.	.	.
6	3	present	none	11	.
7	.	.	.	.	1
8	.	.	.	.	.
9	3	present	none	10	1
10	3	present	present	7	1
11	.	.	.	.	.
12	.	.	.	.	.
13	.	.	.	.	.
14	.	.	.	.	1
15	3	present	present	8	1
16	2	present	present	8	1
17	.	.	.	.	2
18	.	.	.	.	1
19	1	none	none	.	1
20	3	present	none	10	2
21	3	present	none	8	1
22	.	.	.	.	.
23	3	none	none	5	1
24	3	present	none	10	.
25	.	.	.	.	.
26	.	.	.	.	.
27	0	none	none	4	1
28	.	.	.	.	.
29	2	none	none	6	1
30	0	none	present	5	.
31	3	present	present	8	1
32	1	none	present	6	1
33	3	none	present	9	.
34	.	.	.	.	1
35	.	none	none	.	2
36	0	.	.	.	.
37	2	present	present	6	4
38	2	none	none	5	.
39	.	.	.	.	.
40	1	none	none	5	3

F2

subject	gender	BW	actual weight	GA	PCA	alertness
1	male	3.2	5.78	39.3	53	crying
2	female	2.95	4.7	42	49	closed eye
3	male	3.36	5.046	40.4	46.42	closed eye
4	female	3.11	3.969	39.4	42.6	closed eye
5	male	3.35	4.6494	37.3	43	closed eye
6	male	4.082	8	42.1	62.3	open eye
7	male	3.983	6.407	40	53	open eye
8	female	3.92	3.92	41.9	42.27	open eye
9	female	3.94	6.45	41.7	53.14	open eye
10	male	4.337	4.94	38.6	42.4	open eye
11	female	3.49	4.564	40.9	46.14	open eye
12	female	3.32	5.7	38.6	50.85	open eye
13	female	2.79	3.326	39.4	44.57	open eye
14	female	3.05	3.05	39	40	open eye

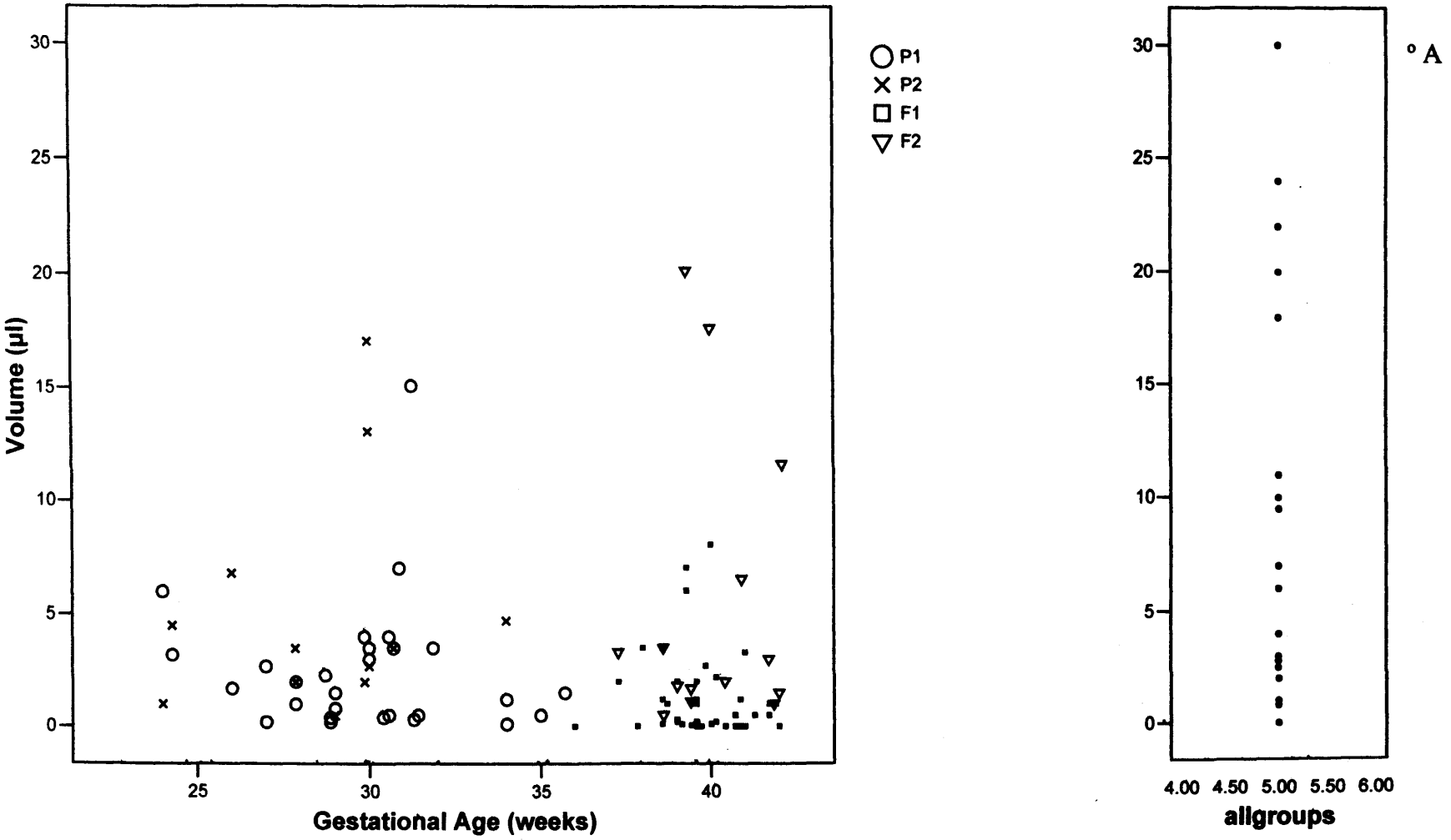
subject	volume (µl)	protein conc. (µg/µl)	IgA (µg/µl)	Lactoferrin (µg/µl)	lysozyme (µg/µl)
1	20	16	0.942	1.016	1.271
2	1.5	17.4	1.535	2.46	5.947
3	2	16.7	1.734	2.865	4.57
4	1.1	.	0.734	1.188	5.176
5	3.3	16.5	2.009	2.874	6.07
6	11.5	13.75	0.389	1.656	4.96
7	17.5	10.65	0.554	1.939	4.1
8	1	7.8	2.017	4.158	5.602
9	3	13.6	0.933	1.181	2.634
10	0.5	.	.	.	.
11	6.5	11.2	1.111	2.926	5.545
12	3.5	6.3	0.223	1.097	1.892
13	1.7	.	0.692	1.543	4.244
14	1.8	7.9	0.803	1.107	1.391

subject	SA (grade)	IgM presence	Lipocalin presence	number of bands	fern (grades)
1	3	present	present	8	.
2	2	none	present	8	.
3	1	present	none	7	3
4	0	none	present	6	2
5	3	present	present	7	1
6	0	none	none	3	.
7	0	none	none	3	.
8	2	present	present	6	.
9	0	none	present	5	1
10	.	.	.	.	3
11	0	none	present	3	3
12	0	none	none	7	2
13	1	none	none	4	1
14	1	none	none	5	1

A

subject	gender	volume (µl)	protein conc. (µg/µl)	IgA (µg/µl)	Lactoferrin (µg/µl)	lysozyme (µg/µl)	Fern (grades)
1	male	2.8	13.8	0.528	1.632	3.596	2
2	female	4	.	3.14	4.8	5.3	.
3	female	4	14.9	2.85	2.74	5.97	.
4	male	18	.	2.2	1.87	0.6	.
5	male	6	14.8	1.051	1.419	3.494	2
6	female	24	10	0.945	1.17	2.912	2
7	female	7	10.6	1.442	1.193	2.273	1
8	female	2	14.1	3.15	2.55	4.15	.
9	female	10	.	1.33	1.4	1.49	.
10	female	22	9.8	1.428	1.411	2.396	0
11	male	20	.	0.94	1.64	1.45	.
12	male	10	9.4	0.874	1.888	3.75	1
13	male	0	.	.	.	.	.
14	female	1	20	.	.	.	1
15	female	9.5	.	1.84	2.36	2.035	.
16	male	11	.	0.49	2.233	1.48	.
17	male	2.5	.	2.58	1.94	1.2	.
18	male	30	.	1.25	1.14	1.88	.
19	male	3	.	1.515	3.045	2.12	0
20	male	6	.	1.68	1.35	2.2	0
21	female	0.8	.	.	.	.	
22	male	4	.	2.18	1.99	2.625	

Tear volume distribution for all subjects in Chapter 5



Appendix 5: Publications

UK). To obtain optimum separation of the cone and rod responses, a red (peak 655 nm) 5 ms flash, was presented at 1.3 Hz over a range of stimulus intensities (0.022–0.264 cds m^{-2}), following 20min dark adaptation and with pupil dilation. In order to mimic conditions of sleep, this was repeated through closed eyelids and without pupil dilation. Finally, background illumination was increased in order to determine the optimum level required to saturate the rod response.

Results: Our technique elicits a clear response from the cone and rod systems simultaneously in the dark adapted eyes of a group of control subjects. Dim ambient illumination suppressed the rod response, whilst the cone response remained intact.

Conclusions: From our preliminary data it appears that levels of 30 lux or less saturate or significantly reduce the rod response, which is presumed to be associated with a marked reduction in oxygen demand.

Reference

Arden G.B., Wolf J.E., Tsang, Y. (1998) Does dark adaptation exacerbate diabetic retinopathy? Evidence and a linking hypothesis. *Vision Res.* **38**, 1723–1729.

The human tear film: sample recovery after collection

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Introduction: The pre-ocular tear film is a fluid with a complex composition and a dynamic structure that has many essential functions for the health of the ocular surface. Any disturbance in the tear film results in loss of stability and dry eye disease and a carefully targeted treatment is difficult. However, the neonate tear film shows a surprisingly high stability and a low blink rate (Lawrenson and Murphy, 2002). Current knowledge about the neonate tear film is limited and further studies of its physiology are needed to explain the observed quality. Less invasive and time-consuming tear collection methods are required for the evaluation of neonates and infants.

Purpose: A sample collection technique needs to be developed that determines materials with good absorbance and extraction properties. Further optimal centrifugation operating modes and possible effects of delayed processing need to be determined.

Methods: Six materials were selected–polyester and cellulose acetate rods, polyvinyl alcohol and cellulose sponges, viscose towels, and Schirmer paper strips. Three different solutions–two high viscosity eye drops and saline–were extracted from the absorbent materials. By measurement of the extracted volume different centrifugation parameters, such as spin duration and speed were tested. Finally many variations of factors, which can alter the extracted volume, such as temperature in the centrifuge, sample volume, receptor size, disinfecting, and delays of 1 h and 7 days, were tested and analysed.

Results: The polyester and cellulose acetate rods and the viscose towel had optimal extraction rates. Centrifugation can be carried out at a setting of 3 min, 14000 rpm and 4°C. Delays enhance the extraction. The small material size and shape helps to obtain more solution out of the rods and altering the sample volume does not make a significant difference. Disinfection had no influence on the structure of the materials.

Discussion: Although the optimal materials were chosen by their extraction rates, we noticed a good absorption when samples were prepared. They need to be tested for the effect of collection on the ocular surface and the effect of extraction on subsequent tear analysis. The extracted amounts were reasonably high so that they might be useful to collect samples for different tear analysis methods in neonates, such as protein analysis, osmolality and ferning testing.

Reference

Lawrenson, J.G. and Murphy P.J. (2002) The relationship between blink parameters, tear film stability and corneal sensitivity in neonates and infants. *Cont. Lens. Ant. Eye.* **25**, 208.

Expression of RhoA mRNA during human corneal transplant rejection

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Purpose: To investigate the mRNA expression of RhoA, a GTPase protein shown to play key roles in immune regulation and apoptosis, during human corneal transplantation.

Methods: A total of 117 blood samples were assessed, 39 of which had successful grafts, and 23 samples showed at least one rejection episode. Fifty-five blood samples were taken as controls showing no ocular conditions. Semi-quantitative analysis was undergone through RT-PCR. One–tailed *t*-test for two samples assuming unequal variances was used to determine the significance of the frequency difference between these groups ($\alpha = 0.05$).

Results: A down-regulation in the mRNA expression of the gene under study was found in the rejecting samples after comparing it to that from successful corneal grafting ($p = 0.024$), whereas the tendency of the rejecting group towards down-regulation was not found to be statistically significant after its comparison with the control group ($p = 0.09$). The difference in expression of RhoA in patients whose condition leading to surgery was herpes simplex keratitis (HSK) was also statistically significant compared with those without HSK ($p = 0.002$).

Conclusion: As the Rho family of GTPases have been proved to take part in the induction of apoptosis, these results might link the low levels of RhoA found in rejectors with a suppression of the apoptotic mechanism against the inflammatory cells that enter the cornea during human allotransplantation, and which would therefore lead to a rejection episode.

Paxillin expression during corneal transplant rejection

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Purpose: To investigate the mRNA expression of paxillin, a focal adhesion protein that recruits adhesion- and growth factor-mediated signals from the extracellular matrix, during human corneal transplantation. Paxillin has previously been shown to play a role in corneal wound repair.

Methods: A total of 125 blood samples were assessed, 45 of which had successful corneal grafts and 25 samples showing at least one corneal rejection episode. The remaining 55 blood samples were used as normal controls, showing no ocular abnormalities. Semi-quantitative analysis was performed by RT-PCR. One-tailed *t*-test for two samples assuming unequal variances was used to determine the significance of the frequency difference between all groups.

Results: mRNA expression of paxillin was found to be significantly down-regulated in the rejecting samples compared with controls ($p = 0.019$). However, no significant difference was found between controls and the non-rejecting group or between subjects in the non-rejecting and rejecting group. Paxillin expression was also found to be significant altered between rejecting and controls or non-rejecting subjects, when age, gender, and surgical risk were compared between these groups.

Conclusion: Our results and previous findings indicate an important role for paxillin during corneal transplant rejection. Low levels of paxillin expression could be responsible for a decreased corneal wound healing response in human allografts, leading to a rejection episode.

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ferred orientation of aligned collagen and the ratio of aligned to total collagen at a known corneal location. By arranging the data onto a grid of corneal position various maps were produced to illustrate the distribution and preferential orientation of collagen. The relationship between collagen arrangement and surface topography was examined in detail for both the normal and keratoconus corneas. **Results:** In keratoconus corneas the orthoganol preferred orientation of collagen fibrils that is seen in the normal human cornea, is absent in the apical region and sometimes beyond. In the normal cornea there is a gradual symmetrical increase of collagen from the central region to the periphery. However, in keratoconus corneas maximal thinning occurs at the apex of the cone. Outside the apical region the increase in collagen occurs asymetrically and is less gradual than in the normal cornea. The distribution of aligned collagen is especially altered in keratoconus corneas and appears to correlate closely with cone shape. **Conclusions:** The results indicate a redistribution of collagen in keratoconus corneas, which supports the theory that corneal thinning in keratoconus occurs as result of lamella sliding away from the apex of the cone. The existence of this mechanism would also help to explain the altered orientation of collagen fibrils in this region.

The Effect of Tear Collection Techniques and Short-Term Storage on the Accuracy of Total Tear Protein

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Purpose: To compare the utility of four different tear collection techniques by monitoring the total tear protein concentrations. The effect of freeze storage on these tear samples was also investigated. **Methods:** Seven subjects were recruited (3 female, 4 male; age range 19–31 years). Six non-stimulated tear samples were taken with each collection method: glass capillary tube, cellulose sponge, polyester rod and cellulose rod. Each collection was completed before noon and limited to a maximum duration of 3 min, with half an hour between samples. Half of the samples were immediately frozen, within the absorbent material, for 7 days at –20°C. To extract the tears, the absorbent materials were centrifuged at 15,000 *g* for 3 min at 4°C, and the tears expelled from the tubes. This sample processing was also repeated for fresh and frozen bovine albumin protein standard (2 g/l). All samples were assayed with the Bradford Coomassie blue test for their total protein content. **Results:** The tear protein concentration was: polyester rod 15.9 ± 1.21 g/l; cellulose rod 12.9 ± 2 g/l; cellulose sponge 12.6 ± 4.1 g/l; and capillary tube 11.9 ± 2.8 g/l. Polyester rod results had the highest concentration for both tears and BSA standard, caused by contaminants in the tubes which affected the assay system. The cellulose rods collected the highest volume of tears (7.6 ± 7.4 µl) and the tube the lowest (2.7 ± 1.3 µl). The BSA standard concentration was increased for the cellulose rod and reduced for the tube ($p < 0.001$) but for the tear protein concentration, no significant difference was shown ($p = 0.17$). BSA and tear samples taken by cellulose rods were not effected by storage ($p_{BSA} = 0.17$ and $p_{tears} = 0.11$). No statistical freeze storage effects were shown for both tears ($p = 0.11$) and standard BSA ($p = 0.47$). **Conclusion:** The cellulose rod offers an ideal alternative to the standard glass capillary tube collection technique.

Tear Lipid Layer Thickness and Ocular Comfort after Use of a Novel Method of Meibomian Therapy in Patients with Dry Eye

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Purpose: To test the efficacy of a novel method of meibomian gland therapy on lipid layer thickness [LLT] and symptoms in patients with dry eye. **Methods:** In a prospective, single masked, randomized, controlled single intervention study 12 patients were treated with the device and 12 underwent no treatment. LLT and dry eye symptom score were assessed before, initially after and 30 min after intervention. **Results:** For treated eyes LLT increased by 1.2 levels for right and 1.0 level for left initially ($p < 0.0005$), and 0.7 for right and 0.6 for left after 30 min ($p < 0.005$). Symptom scores were significantly improved in the treatment group initially ($p < 0.05$) and 30 min after intervention ($p < 0.02$) compared with the control group. **Conclusions:** This device increases LLT and reduces symptoms in patients with dry eyes.

Recombinant Keratoepithelin Expression and Its Effects on Corneal Epithelial Cells

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Purpose: Keratoepithelin is an extracellular matrix protein associated with a number of corneal dystrophies. The aim of this study was to express native wild type and mutant recombinant keratoepithelin and study its effects on primary human corneal epithelial cells. **Methods:** βig-h3, the gene that encodes for keratoepithelin, was subjected in vitro mutagenesis, to generate two pathological mutant forms. The mutations were verified by DNA sequencing and the three genes were subsequently cloned in a prokaryotic expression vector and were expressed in *E.coli.* Purification was mediated with Ni-NTA His-bind resin. The effects of recombinant proteins on the adhesion, migration and apoptosis of corneal epithelial cells were assessed. **Results:** Mutagenesis gave rise to R555Q and R555W clones, which correspond to Thiel Behnke and granular corneal dystrophies respectively. The recombinant proteins were expressed both in the soluble and insoluble part of the cellular extract, as determined by SDS-PAGE and Western blotting. Purification took place under native conditions and the resulting average concentration was 1 mg/ml from a 50-ml culture. The recombinant proteins did not have a significant effect on corneal epithelial cell migration. However the R555W mutant appeared to alter cell adhesion. **Conclusion:** The in vitro studies on human primary corneal epithelial cells indicate that native recombinant keratoepithelin can provide a useful insight in the pathology of the anterior corneal dystrophies.

Tear Film & Ocular Society conference abstract 2004

THE EFFECT OF TEAR COLLECTION TECHNIQUE ON THE ACCURACY OF TOTAL TEAR PROTEIN AND SERUM ALBUMIN

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Purpose. To compare the accuracy of two different tear collection techniques by monitoring the concentrations of standard bovine serum albumin (BSA) and total tear protein. To assess their safety of use by measuring serum albumin (SA) leakage from the conjunctiva. To investigate their volume absorbance efficiency. **Methods**. Seven subjects were recruited (3 female, 4 male; age = 19-31ys). Two non-stimulated tear samples were taken, one with a glass capillary tube (CT) and one with a cellulose rod (CR), on three different days. Each collection was completed before noon and limited to a maximum duration of 3min, with 1/2h between samples. To extract the tears, the cellulose rod was centrifuged at 15000g/3min/4°C, and the tears expelled from the tubes. All samples were assayed with the Bradford test for their total protein content. In a second study, 27 subjects (17 female, 10 male; age = 22-35ys) were recruited. One tear sample was taken with each technique over a maximum time of 1min 45sec. Samples were subjected toSDS-PAGE and stained with silver stain. The SA band intensities were measured and compared to a commercial human SA as standard (0.25g/l). **Results.** The average tear protein concentrations found were: CR 12.9g/l ±2; CT 11.9g/l ±2.8. The average tear volumes collected were: CR 7.6µl ±7.4; CT 2.7µl ±1.3. The BSA concentration measured for the CR was increased from the actual inserted concentration, due to volume changes from absorption and evaporation effects. The concentration was reduced for the CT. This produced a significant difference between the two techniques ($p<0.001$). However, for the tear protein concentration no significant difference was shown ($p=0.17$), because of higher volumes and variation in subjects. In the second study, 22 subjects had at least 0.5µl of tears collected with both techniques. Six subjects had SA at measurable levels, but no difference ($p=0.526$) was found between the techniques. In the remaining samples SA levels were below the quantification limit. For all subjects there was no clinical significant increase to prove serum leakage. **Conclusion**. The cellulose rod offers an ideal alternative to the standard glass capillary tube.

ARVO abstract 2005

Program#/Poster#:	4398/B756
Abstract Title:	**The Effect of Tear Collection Technique on the Accuracy of Total Tear Protein, Major Proteins, and Serum Albumin**
Presentation Start/End Time:	Wednesday, May 04, 2005, 11:15 AM - 1:00 PM
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Author Block:	*M.Esmaeelpour, M.Boulton, J.Cai, P.J. Murphy.* Optometry & Vision Science, Cardiff University, Cardiff, United Kingdom.
Keywords:	481 cornea: tears/tear film/dry eye

Purpose: To compare the accuracy of two different tear collection techniques by monitoring the concentrations of standard bovine serum albumin (BSA) and total tear protein. To determine the effect of collection method on the major tear proteins: IgA, lactoferrin (LF), lysozyme (LZ) and serum albumin (SA). To investigate their volume absorbance efficiency.
Methods: Seven subjects were recruited (3 female, 4 male; age = 19-31ys). Two non-stimulated tear samples were taken, one with a glass capillary tube (CT) and one with a cellulose rod (CR), on three different days. Each collection was completed before noon and limited to a maximum duration of 3min, with 1/2h between samples. To extract the tears, the cellulose rod was centrifuged at 15000g/3min/4°C, and the tears expelled from the tubes. All samples were assayed with the Bradford test for their total protein content. In a second study, 27 subjects (17 female, 10 male; age = 22-35ys) were recruited. One tear sample was taken with each technique over a maximum time of 1min 45sec. Samples were run subjected to onSDS-PAGE and visualizedstained by with silver stain. For the major tear protein study, 16 subjects (7 female, 9 male; age = 22-31ys) were recruited. Tear samples were taken similar to the second study but gels were stained by blue stain. Commercial standards used were human SA (0.25g/l), IgA (1.8g/l), LF (2.5g/l), and egg LZ (2.5g/l).
Results: The average tear protein concentrations found were: CR 12.9g/l ±2; CT 11.9g/l ±2.8. The average tear volumes collected were: CR 7.6µl ±7.4; CT 2.7µl ±1.3. The BSA concentration measured for the CR was increased from the actual inserted concentration, due to volume changes from absorption and evaporation effects. The concentration was reduced for the CT. This produced a significant difference between the two techniques ($p<0.001$). However, for the tear protein concentration no significant difference was shown ($p=0.17$), because of higher volumes and variation in subjects. In the second study, 22 subjects had at least 0.5µl of tears collected with both techniques. For all subjects there was no clinical significant increase to prove serum leakage. Results for major tear proteins were obtained from 12 subjects. No significant difference was shown between both collection methods for IgA ($p=0.45$), LF ($p=0.22$) and LZ ($p=0.29$) although significantly higher volumes were collected with the CR ($p=0.004$).
Conclusions:
The cellulose rod offers a suitable alternative to the standard glass capillary tube.

Commercial Relationship:	**M. Esmaeelpour**, None; **M. Boulton**, None; **J. Cai**, None; **P.J. Murphy**, None.
Support:	None.

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ARVO abstract 2006

Program#/Poster#:	1942/B392
Abstract Title:	**Tear Volume and Protein Concentration in Premature and Full-Term Infants**
Presentation Start/End Time:	Monday, May 01, 2006, 3:00 PM - 4:45 PM
Location:	Hall B/C
Reviewing Code:	209 lacrimal gland and tear film - CO
Author Block:	*M.Esmaeelpour[1], P.O. Watts[2], J.Cai[1], M.Boulton[1], P.J. Murphy[1].* [1]School of Optometry and Vision Sciences, Cardiff University, Cardiff, United Kingdom; [2]University Hospital of Wales, University of Wales College of Medicine, Cardiff, United Kingdom.
Keywords:	481 cornea: tears/tear film/dry eye

Purpose: To investigate the development of normal neonatal tear volume and protein concentration, with age and weight.
Methods: Two experimental groups were recruited: 30 premature infants (14 female, 16 male; post-conception age (PCA) 35.77±1.77 weeks, 1.87±0.54kg) and 40 full-term infants (19 female, 21 male; 39.86±1.27 weeks, 3.42±0.53kg). As a control, 22 adults (10 female, 12 male; age 24.95±3.63 years) were also recruited. One non-stimulated sample was taken from the lower tear meniscus of one eye using a cellulose rod. This tear collection technique is non-invasive and ensures minimum stimulation of the ocular surface. Tear collection was repeated for 12 premature infants (7 female, 5 male; 37.19±1.76 weeks, 1.97±0.43kg) and 14 full-term infants (8 female, 6 male; 47.76±6.14 weeks, 5.04±1.34kg). Where volume permitted, samples were assayed with the Bradford test for their total protein content.
Results: The mean tear volume (µl ±sd) in premature infants was 2.25±1.28 (6/30 samples <0.5µl) and in full-term infants 2.70±2.29 (9/40 no tear sample detected, 9/40 samples <0.5µl). A repeated sample from the premature group was 5.52±4.84 (all >0.5µl) and from repeated full-term infants 4.51±6.09 (all >0.5µl). In adults the tear volume was 8.95±8.54 (1/22 no tear sample detected). No difference was found between the premature and full-term infant groups (Tukey, $p>0.05$). Tear volume increased significantly for the repeated tear collection and approached adult like levels (Tukey, $p>0.05$). However, all infant group volumes were still lower than the adult's tear volume (ANOVA, $p<0.001$). Mean total tear protein concentrations (g/l ±sd) was 12.43± 4.32 in premature infants and 10.95± 5.51 in the full-term infants. The repeated premature group was 14.84± 4.29 and 12.93± 3.99 in the repeated full-term infants. In adults the total protein was 13.04± 3.46. No significant difference was found in protein concentrations between any of the groups (ANOVA, $p>0.05$).
Conclusions: 1) Premature and full-term infants have a lower tear volume than adults; 2) No difference between premature and full-term infant tear volumes; 3) Tear volume increases rapidly after birth; 4) No differences in total tear protein concentration, despite volume differences.

Commercial Relationship:	**M. Esmaeelpour**, None; **P.O. Watts**, None; **J. Cai**, None; **M. Boulton**, None; **P.J. Murphy**, None.
Support:	None

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The neonatal tear film

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Abstract

The importance of the tear film for the integrity of the ocular surface is well established. Full-term neonates produce tears normally, but low spontaneous blink rates during early life raises important questions regarding tear dynamics and stability. Although an afferent neural pathway that could potentially detect tear break-up is in place at birth, there is indirect evidence that the neonatal tear film is adapted to resist evaporation-mediated tear thinning. This adaptation presumably prevents drying of the ocular surface during long inter-blink periods. However, low rates of tear turnover may have important implications for the defence of the eye against potential pathogens.
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Keywords: Neonates; Tear film; Lipid layer; Blinking; Corneal sensitivity

1. Introduction

The ocular surface, together with the eyelids and lacrimal system, represents a functional unit that ensures the quality of the refractive surface of the eye and also protects ocular structures from noxious stimuli arising from the environment [1]. The pre-ocular tear film is the most dynamic structure within this unit, and an adequate rate of tear production and a rapid turnover is essential for ocular health. Tears cleanse, lubricate and defend the ocular surface against infection. Furthermore, by smoothing out irregularities of the corneal epithelium the pre-corneal tear film creates an even surface of excellent optical quality that is reformed with each blink. In the newborn, the optical characteristics of the tear film are important for the generation of a clear retinal image, which is an essential pre-requisite for normal visual development. Since the tear film is inherently unstable, frequent blinking is normally required to prevent surface drying. The reported low rate of spontaneous blinking in neonates and infants [2,3] thus raises important questions regarding tear stability and the ability of ocular surface receptors to detect tear break-up. This review will summarise our current knowledge of several aspects of the neonatal tear film and will examine the relationship between blink parameters, tear dynamics and corneal sensation.

2. Tear production during the neonatal period

For many years, there was a general belief that lacrimation was absent or minimal during the first few weeks of life [4]. However, Sjögren was able to show that a lacrimal reflex was present in the majority of newborns, although tear secretion was generally sub-normal [5]. Subsequent studies using the Schirmer test [6–9] have shown that the majority of full-term neonates do in fact possess tear production levels comparable to adults (Table 1). Normal tearing, defined as at least 15 mm of wetting of the test strip in 5 min, is present in over 80% of babies at birth, and in almost all by 1 month. By contrast, pre-term babies show reduced rates of tear secretion, and total tear production correlates with the maturity parameters of birth weight and post-conceptional age. A recent study found that only 10% of premature babies secreted tears normally [9], and the remainder showed reduced rates of basal and reflex tearing. This is significant since a reduced rate of reflex secretion may limit the dilution of topical ocular medication, leading to longer contact of the drug with the ocular surface, and a greater risk of adverse reactions [8].

3. Patency of the nasolacrimal system

In the foetus, the nasolacrimal duct forms initially as a solid mass of cells that becomes canalised late in development. At birth, the duct lumen is markedly irregular with numerous folds and crypts [11] and often fails to completely canalise [12]. Most commonly, occlusion occurs at

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Table 1
Rate of tear secretion for term and pre-term neonates

Age range (post-conception)	Basal secretion (mm)	Reflex secretion (mm)	Reference
Term			
38–42 weeks	9.2	13.2	[8]
38–42 weeks	7.3	9.3	[9]
Pre-term			
30–38 weeks	6.2	7.4	[8]
28–37 weeks	7.7	3.5	[9]
Adults	7.8	17.5	[10]

Carrying out the Schirmer test with and without anaesthetic differentiates between basal and reflex secretion.

the extreme distal end of the duct just before it empties into the nasal cavity (Fig. 1). In a large prospective study of neonates and infants during the first year of life, 20% of eyes showed evidence of symptomatic occlusion [12]. Typical symptoms included watering and mucopurulent discharge. However, there is a high rate of spontaneous canalisation, and 70% of affected children are symptom-free by 3 months of age, and over 90% by their first birthday [13].

A fluorescein disappearance test is useful in the differential diagnosis of an infant presenting with persistent epiphora and discharge [12]. A drop of 1% fluorescein is instilled into the lower fornix and the eye is examined in blue light 5–10 min later. Complete clearance of the dye indicates infants with a normal nasolacrimal drainage system. Retention of the dye within the tear film is indicative of obstruction.

Management of congenital nasolacrimal duct obstruction should be conservative in the first instance, since most cases resolve during the first year of life [12]. Treatment consists of simple lid hygiene coupled with gentle massage of the lacrimal sac [12]. Topical antibiotics should only be given where there is clinical evidence of infection. In the UK, nasolacrimal duct probing under general anaesthesia is the intervention of choice in cases that have not resolved by 1 year. The treatment has a 90–95% success rate when performed in the first 2 years of life, but is less effective in older children [12].

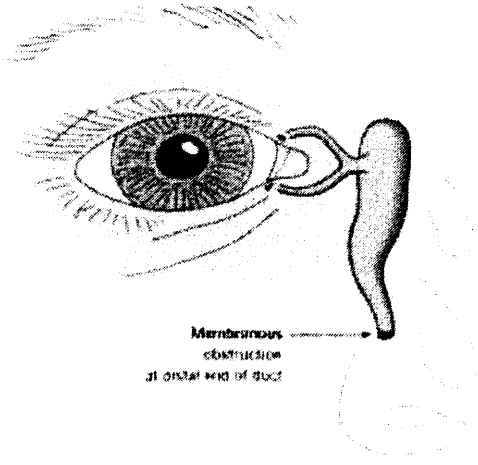

Fig. 1. Congenital obstruction of the nasolacrimal duct. The site of obstruction is typically at the distal end of the duct. Taken from [12].

4. Factors affecting tear dynamics and stability

4.1. Blinking

Spontaneous blinking plays an important role in the spreading of the tear film across the ocular surface, and also drives tears through the nasolacrimal ductal system. However, the mechanisms involved in the control of spontaneous blinking are not fully understood, although it is likely to involve the interplay between central and peripheral factors. There is clear evidence that the central nervous system is important for blink control, for example, blink rates are highly influenced by attentional and cognitive states, and electrical stimulation of several cortical and subcortical areas can elicit blinking [14]. A role for peripheral factors has only recently been demonstrated, and there is accumulating evidence that local ocular surface reflexes, and the environmental factors that affect them, have a major influence on blink rate [15].

Whilst reflex blinking is well developed at birth, early observers claimed that spontaneous blinking only appears at 6 months, and increases in frequency thereafter [2]. However, more recent studies have recorded low rates of spontaneous blinking in very young infants [3,16]. We have recently carried out a systematic analysis of blink rates and inter-blink periods during the first year of life (Fig. 2). Newborns typically blink between two and three times per minute, although inter-blink times in excess of 1 min are commonly seen. Spontaneous blink rates continue to increase rapidly during the first year, although still remain lower than published values for adults, which lie in the range of 12–20 blinks per minute [15,17]. This corresponds to a blink every 3–5 s.

The observation of a low spontaneous blink rate in neonates and infants is significant. One possible explanation

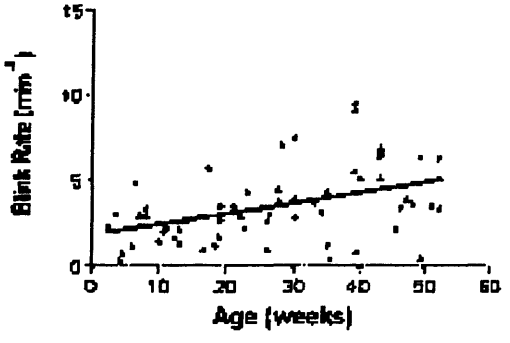

Fig. 2. Scatter plot showing a significant correlation between blink rate and age over the first year of life ($N = 64$, $P = 0.0002$).

is that at this stage of development the neural circuits for blink control are still relatively immature. Nevertheless, given the inherent instability of the tear film, one would expect that such long inter-blink periods would compromise ocular health and retinal image clarity, unless the neonatal film is adapted to remain stable for long periods, which would obviate the requirement for frequent blinking.

4.2. Factors affecting tear evaporation

Although the bulk of the tear volume drains through the nasolacrimal system, a significant proportion of tears are lost to evaporation. Conditions that increase tear evaporation, such as an increase in exposed ocular surface area, air-flow across the ocular surface or evaporative dry eye, are associated with increased blink rates and reduced inter-blink times [18,19]. The relationship between exposed ocular surface area and tear evaporation is particularly pertinent. The tear evaporation rate is 3.4 and 2.5 times higher when looking up and straight ahead compared with down [20]; moreover, the performance of near vision tasks in down gaze is associated with significantly reduced blink rates [21]. Whilst we may intuitively suppose that neonates possess a small exposed ocular surface area, the development of the palpebral aperture during the post-natal period has only received sparse attention. Quantitatively, the aperture can be defined in terms of its horizontal length and vertical width (Fig. 3). The horizontal dimension is usually measured from canthus to canthus, whilst the vertical width is taken at the widest point between the lids with the eyes in the primary position.

In Caucasians under 1 year of age, the vertical to horizontal palpebral aperture ratio is approximately 1:2, increasing to a value of 1:3 in adults due to a proportionally greater increase in the horizontal length with age [22]. The consensus of opinion is that the greatest change in palpebral dimensions occurs during the first year of life [23,24]. We have recently investigated palpebral aperture dimensions in neonates and infants (Fig. 4), and confirmed that a rapid increase in width and length occurs from birth to 1 year. The rate of change was greatest for the horizontal dimension.

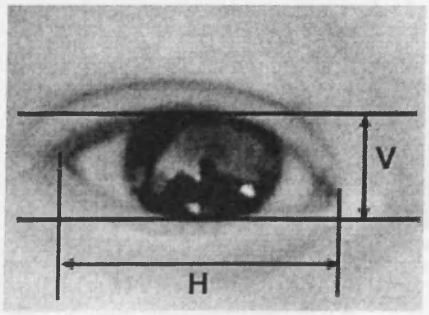

Fig. 3. Measurement of horizontal and vertical palpebral aperture dimensions.

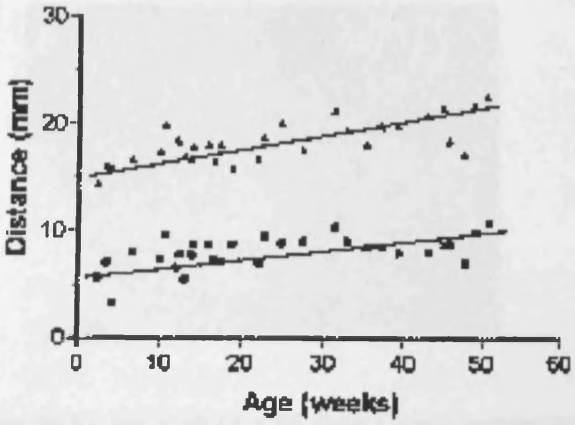

Fig. 4. Variation in vertical width (lower graph) and horizontal length (upper graph) with age in Caucasians during the first year of life. Both measurements are highly correlated with age ($N = 30$, $P < 0.001$).

These changes in palpebral aperture size have a particularly dramatic effect on the exposed ocular surface area. When subjects are grouped into age bands (Fig. 5), a 50% increase in mean surface is observed from 0–17 to 36–53 weeks.

These differences in area are likely to have a significant impact on the rate of tear evaporation and blink rate. It is reasonable to assume that as the palpebral aperture increases the lipid layer thins, which would be associated with a greater probability of tear break-up and a compensatory increase in blink rate [15].

The importance of the tear lipid layer for retarding evaporation and maintaining tear stability is well documented [25–28]. The lipid layer can be readily imaged by non-invasive interferometric methods, which provide qualitative and semi-quantitative information about the integrity and thickness of the layer [29]. Using this technique, it has been shown that thick lipid layers are associated with a reduced rate of evaporation and longer tear break-up

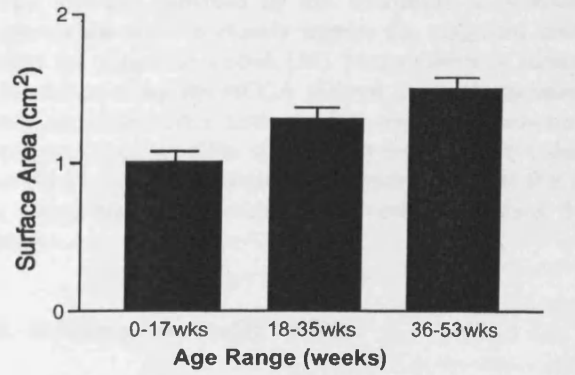

Fig. 5. Variation in exposed ocular surface area with age ($N = 30$). Exposed ocular surface area was calculated using the method described by Zaman et al. [16]. The difference between the 0–17 and the 36–53 week groups is statistically significant ($P < 0.01$).

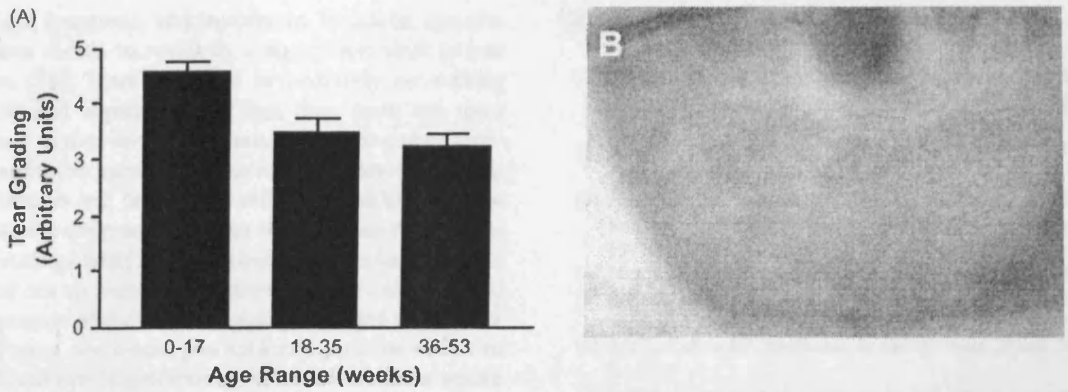

Fig. 6. (A) Graph showing the variation in Tearscope tear grading with age over the first year of life ($N = 64$). Grade 1 = open meshwork pattern, Grade 2 = wave pattern, Grade 3 = amorphous pattern, Grade 4 = colour fringe pattern covering part of the tear film, Grade 5 = colour fringe pattern covering the majority of the tear film. (B) Tearscope image showing the colour fringe pattern that is typical of neonatal lipid films.

times compared to thin films [26–28]. Significantly, we have observed that the neonates show a high incidence of interference patterns typical of thick lipid films, and in the first few months of life a colour fringe pattern predominates [16] (Fig. 6).

It is not yet clear whether the greater lipid layer thickness in neonates is related to a small palpebral aperture or to inherent biophysical differences of the lipid components. However, it may be significant that meibomian lipids, collected from infants aged 1.5 years, show a greater ability to withstand repeated compression and expansion than equivalent samples taken from adults [30]. It is possible that the superior biophysical characteristics of infant lipids may be a further adaptation to compensate for a reduced blink rate. The importance of blinking for the transfer of lipids from the glandular acini and ducts to the lid margin reservoir is well documented [31]. The action of the orbicularis during blinking applies a contractile force to the duct and acini so that with each blink a "jet" of oil is expressed from each duct orifice. In neonates it is envisaged that because of ductal accumulation of meibomian secretion between blinks, the output per blink would be greater than in adults.

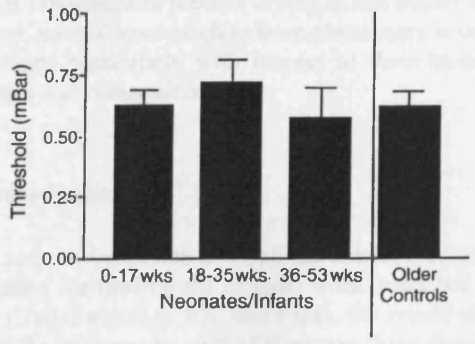

Fig. 7. Corneal sensation threshold for neonates compared to a group of older subjects.

4.3. Development of an afferent pathway for the detection of tear break-up

Infrared radiation thermography has shown an evaporation-mediated fall in corneal temperature between blinks [32]. The adult cornea is well equipped with thermosensitive receptors to detect such changes in surface temperature [33], and a model has been proposed whereby cold receptors may initiate a blink by signalling evaporative cooling [32]. The importance of this afferent pathway is emphasised by the fact that blink rates are significantly reduced following topical anaesthesia, where discharge from corneal receptors is inhibited [34,19]. We wanted to address the question as to whether neonates possess the necessary neural machinery for the detection of tear break-up. Standard methodology for the measurement of corneal sensitivity in humans typically utilises invasive mechanical stimuli [35]; however, a non-contact corneal aesthesiometer (NCCA) has been developed which produces a localised cooling of the corneal surface [36,37], and at the same time ensures that there is no risk of damaging the corneal epithelium. This stimulus provided by this instrument is particularly appropriate since it closely mimics the suggested mechanism for triggering a blink [32]. Measurement of sensation thresholds using the NCCA showed that a thermosensory reflex is present from birth, and that sensation thresholds for neonates does not differ significantly from those recorded in adults [16] (Fig. 7). These data therefore suggests that that a system for the detection of tear break-up is present from birth.

5. Defence of the ocular surface

Tears play a major role in the defence of the eye against infection. The washing action of the tear fluid reduces the likelihood of microbial adhesion to the ocular surface; moreover, tears contain a host of protective antimicrobial proteins,

including IgA, lysozyme, and lactoferrin. In adults, eye closure has been shown to result in a significant shift in tear composition [38]. Tears collected immediately on waking contain a 50-fold higher level of IgA than open eye tears and also show higher levels of reactive complement components and activated polymorphonuclear leukocytes (PMN). These differences are thought to reflect the shift from the open eye passive defence state to an active inflammatory environment during sleep [38]. The equivalent studies have not been carried out on neonates. However, since babies spend a large proportion of their time sleeping, coupled with a low turnover of tears, one would predict a composition closer to the adult closed eye. Significantly, newborn tears are acidic [39] which is similar to that found in adults following prolonged eye closure [40]. This shift has been attributed to increased anaerobic metabolism of the cornea, but may also reflect PMN recruitment, since these cells are highly anaerobic [38]. The available data on the levels of secretory IgA (sIgA) in neonatal tears is sparse, although evidence points to the fact that the ocular secretory immune system may not be fully mature at birth [7,41,42]. Any potential deficiency in sIgA may be offset by the presence of lysozyme, whose levels in full-term neonates appear to be equivalent to those found in adults [7,43]. By contrast, pre-term babies may show reduced levels of antimicrobial proteins [43], and as a result may therefore more vulnerable to infection. Further research is needed to address these important issues.

6. Conclusions

Compared to wealth of information on the pre-ocular tear film in adults, our knowledge of the neonatal tear film is limited. It has now been finally established that neonates produce tears normally, although the low blink rate in early life would suggest that the turnover of tears is low. The neonate seems to be adapted for prolonged periods of eye opening. The finding of a high incidence of interference patterns associated with a thick and stable lipid layer would appear to be an adaptation to resist evaporation mediated tear film thinning, and prevent drying of the ocular surface. However, we still have much to learn about tears in neonates and infants, particularly with respect to their biophysical properties and composition.

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