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Development of a New Lubricant and Nutrient Tear Substitute

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1 INTRODUCTION

1.1 THE OCULAR SURFACE

1.1.1 Anatomy of the ocular surface

The ocular surface, including cornea and conjunctiva, and the tear film form a functional unit. The cornea is composed of five layers which are epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium. The transparency of the cornea is due to its uniform structure, avascularity, and deturgescence. The corneal epithelial stem cells reside in the basal layer of the peripheral cornea in the limbal zone. These cells have superior proliferative capacity compared to the central corneal epithelial cells, therefore, they provide the potential for rescue or reconstruction of the damaged corneal epithelium (Sun and Lavker 2004). The conjunctiva is the thin, transparent mucous membrane overlying the sclera. It has three parts - palpebral, forniceal and bulbar - and histologically consists of epithelium and stroma. There are numerous other cell types resident within the epithelium besides epithelial cells, such as goblet cells, melanocytes, langerhans´ cells and lymphocytes. Goblet cells are responsible for the secretion of the majority of conjunctival mucins. The accessory lacrimal glands of Krause and Wolfring are located in conjunctival stroma and are thought to be accountable for the baseline tear production. (Forrester, Dick et al. 2002)

The corneal and conjunctiva epithelia have different cytokeratins (CKs) patterns. Cytokeratins are the most complex group within the intermediate filament family and are present in almost all invertebrates epithelial cells. Keratins exist in a 1: 1 ratio type I (acidic, including CK9 to CK20) and type II (basic, including CK1 to CK8). The keratins of the corneal epithelium have been shown to be composed of a major keratin pair, formed by acid keratin, CK12, and a basic keratin, CK3, together with a minor keratin pair, acid CK14 and basic CK5. The types of keratins synthesized are specific to the development of the stage and the phenotype of the cells (Moll, Franke et al. 1982). Cytokeratins characteristic of nonkeratinized, stratified (CK4 and CK13), simple (CK8 and CK19), and glandular epithelia (CK7) were present in the superficial layer of normal human conjunctival epithelium (Krenzer and Freddo 1997).

1.1.2 Nutrition of the ocular surface

Fundamental to the wetting of the ocular surface is the nature of the epithelial cell membrane. Membrane bound subsurface vesicles that contain glycoproteins in the conjunctival epithelium rise to the tear-side surface of the cell and fuse with the cell membrane to distribute glycoproteins over the epithelial cell surface, which contributes to the maintenance and retention of an evenly distributed mucous layer. To maintain the cellular activities, adenosine triphosphate (ATP) is required as an energy source. Generally, degradation of glucose through glycolysis generates ATP under aerobic conditions. Therefore, glucose and oxygen are essential to maintain the normal functions of the cornea. Glucose is supplied by diffusion from the aqueous humor and oxygen requirements of ocular surface are met by the diffusion from the tear film under open eye conditions and from the lid vasculature in closed eye conditions (Krachmer, Mannis et al. 1997) (Harding 1997).

The ocular surface is covered by tear fluid and can't retain its normal structure in the absence of a normal tear film. The tear film serves not only as a lubricant and nutritional source for the corneal epithelium, but also as the source of the regulatory factors for the maintenance and repair of the corneal epithelium, since it contains a wide range of various biologically active substances such as hormones, growth factors, cytokines and reciprocal receptors (Wilson, Li et al. 1998) (Nakamura, Sotozono et al. 1998) (Wickham, Gao et al. 2000). Some of these factors such as epidermal growth factor (EGF) and interleukin 6 (IL 6) have been reported to modulate corneal epithelial migration, proliferation, and differentiation (Watanabe, Nakagawa et al. 1987) (Nishida, Nakamura et al. 1992). Normal maintenance and growth of ocular surface epithelia is dependent on an adequate supply of vitamin A, and its deficiency can result in xerosis of the ocular surface (Pfister and Burstein 1976) (Sommer 1998).

The deficiency of nutrition for ocular surface frequently leads to metaplasia of the surface epithelia with potential risk for rescue failure of the stem cells and conjunctivalisation of the corneal epithelium, with associated loss of vision due to the poor optical quality of the conjunctival epithelium. The most common cause of ocular surface disease remains the dry eye.

1.2 DRY EYE SYNDROME

1.2.1 Function and composition of tears

The production and turnover of tears is essential for maintaining the health of the ocular surface. Tears clean, lubricate and nourish the surface of the eye, and provide physical and immune protection against infection and mechanical trauma. The tear film is composed of three main components, mucin, water, and lipid. More than 98% of the total tear volume is water. The average thickness of the tear film varies between 4.0 and 9.0 µm (Maurice 1967). The 0.02-0.05 µm thin inner mucin layer is produced by conjunctival goblet cells and epithelial cells of conjunctiva and cornea. It plays an important role in tear spreading; the 6-7 µm thin aqueous layer is produced by the main and accessory lacrimal glands. It is responsible for carrying essential growth factors to the epithelium and washing away the epithelial debris, toxic elements and foreign bodies; the outer 0.1 µm thin lipid layer is produced by meibomian glands and makes an important contribution to prevent the evaporation of tears.

Tears are also composed of electrolytes, proteins, growth factors, vitamins, amino acids and glucose besides water, mucus and lipids. The composition of tears resembles that of serum. Table 1 shows the concentrations of the major components of tears and serum (Tsubota and Higuchi 2000). Tears contain the same range of electrolytes as blood plasma, but with characteristic differences, i.e. higher level K^+ and lower level Na^+. Lysozyme, the major protein in tears, is markedly higher than that in serum. IgA is the major immunoglobulin in tears and responsible for defence against infections of the ocular surface. The concentrations of most growth factors between tears and serum are equivalent, with the exception of TGF-ß1, which is found at much lower concentrations in tears than in serum. Tears contain lower level of vitamin A but more vitamin C than serum. There are also a variety of cells in the tear film, including squamous cell from corneal and conjunctival epithelium, lymphocytes and plasma cells from capillaries and the lymphoid system of the conjunctiva.

Table 1: Components of human tears and serum

	Concentration	
Components	**Tears (basal)**	**Serum**
Electrolytes		
Na^+	145 mEq/L	135-146 mEq/L
K^+	24.1 mEq/L	3.5-5.0 mEq/L
Ca^{2+}	1.5 mM	1.1 mM
Cl^-	128 mM	96-108 mM
Proteins		
Total protein	7.37 g/L	68-82 g/L
Lysozyme	2.39 g/L	4.0-15 mg/L
Lactoferrin	1.51 g/L	ND
Albumin	54 g/L	35-55 g/L
IgA	411 mg/L	0.9-4.5 g/L
IgG	32 mg/L	8-18 g/L
Growth factors		
EGF	1.66 ng/L	0.72 ng/L
TGF-α	247 pg/L	147 pg/L
TGF-ß1	ND	140.3 pg/L
TGF-ß2	55 pg/L	-
Vitamins		
Vitamin A	16 ng/L	883 ng/L
Vitamin C	117 µg/L	7-20 µg/L
Antioxidants		
Tyrosine	45 µM	77 µM
Glutathione	107 µM	ND
Glucose	26 mg/L	0.6-1.2 g/L

ND = not detected

1.2.2 Introduction to dry eye

Kerotoconjunctivitis sicca (KCS) or dry eye is a disorder of the tear film due to tear deficiency or excessive evaporation which causes damage of the interpalpebral ocular surface and is associated with symptoms of discomfort. The definition of dry eye has been expanded to include any functional or component anomaly of the lids or a gland associated with tear production in which the quality and/or quantity of the tear film is adversely affected and there is an inability to maintain a healthy ocular surface (Albietz 2001). Dry eye disease is among the most frequently established diagnosis in ophthalmology. Data on the epidemiology of dry eye are varying because of the variation in the definition and diagnostic techniques. Hikichi showed that of 2127 outpatients of 8 Japanese centres, 17%

were suffering from symptoms for dry eye (Hikichi, Yoshida et al. 1995). Schein reported that in 2240 noninstitutionalized residents based studies symptoms of dry eye are found in up to 14.6%, while ocular surface changes are observed clinically in only 0.5% (Schein, Tielsch et al. 1997).

Clinically dry eye disease can be assigned to two major classes: aqueous deficient dry eye, due to a reduced aqueous tear secretion; and evaporative dry eye. *Decreased tear secretion* may result from any condition that damages the lacrimal gland or its excretory ducts. Autoimmune disease with inflammation of the lacrimal gland such as Sjögren's syndrome is the most common cause. Less common causes are cicatricial ocular surface condition and any condition that decreases corneal sensation, including diabetes, herpes zoster, long-term contact lens wear and surgery that involves corneal incisions or ablates corneal nerves. *Increased tear evaporation* may occur in one of two ways: 1) Long-standing posterior blepharitis causing meibomian gland dysfunction. The meibomiam glands and lacrimal glands appear to require androgens to support their normal function (Azzarolo, Mircheff et al. 1997), and androgen deficiency may promote meibomian gland dysfunction and evaporative dry eye (Sullivan, Sullivan et al. 2002). 2) A large palpebral fissure width, occurring either naturally, secondary to cosmetic surgery or with thyroid eye disease, places evaporative stress on the tear film. Evaporation is proportional to the palpebral-fissure surface area. Increased evaporation also explains why symptoms become worse with exposure to air conditioning, dry heat, low humidity or wind (Albietz 2001).

Whatever the initial cause of dry eye, chronic dryness of the ocular surface results in an inflammatory reaction which is the key mechanism of chronic ocular surface injury. The global features in common for both forms of dry eye are: 1) a set of characteristic symptoms, 2) ocular surface damage, 3) reduced tear film stability, and 4) tear hyperosmolarity (Baudouin 2001). The patients often complain about grittiness, foreign body sensation, burning, soreness, stinging scratchiness, dryness, blurry vision, a 'film over the eyes', paradoxical reflex tearing, and photophobia. Absolute tear deficiency can lead to blindness due to severe ocular surface disease and attempts of surgical reconstruction frequently fail in this situation (Geerling, Liu et al. 2002; Geerling, Liu et al. 2003).

1.2.3 Therapy of dry eye

The goals of dry eye treatment are to reduce symptoms, to improve tear film quantity and quality and to reverse ocular surface damage. Therapeutical approaches include (1) tear substitution, (2) pharmacological stimulation of tear secretion and (3) tear preservation through reduction of tear evaporation or drainage (Calonge 2001).

Tear substitutes, the most widely used therapy for dry eye, are composed of a blend of polymers, electrolytes and buffering systems. An ever increasing number of artificial tear formulations are available on the market with different therapeutic characteristics. Cellulose ethers have good retention time on the ocular surface (Bach, Adam et al. 1972), sodium hyaluronate has been found particularly beneficial in corneal wound healing (Sugiyama, Miyauchi et al. 1991; Shimmura, Ono et al. 1995), carbomers provide excellent adhesive behaviour and higher retention time (Sullivan, McCurrach et al. 1997) and recently lipid containing drops aim to re-build the lipid layer (Rieger 1990). Alternatively natural tear substitutes, such as autologous serum or saliva, have been found beneficial in severe dry eye (Fox, Chan et al. 1984; Macleod, Kumar et al. 1990; Tseng and Tsubota 1997; Geerling, Sieg et al. 1998). They help epithelial defects heal and improve the ocular surface because of their lubricating ability, natural viscosity, and nutritive substances that are also found in tears such as EGF, vitamin A or fibronectin (Schultz, Davis et al. 1988) (Tsubota, Goto et al. 1999). But their time consuming production and biological origin prevent them to become off the shelf, quality controlled solutions for widespread use in ocular surface disease.

Stimulation of lacrimal secretions with systemic pilocarpine, a muscarinic M3 agonist, improves subjective and objective assessment of Sjögren's syndrome (Tsifetaki, Kitsos et al. 2003). Several topical medications have been found to be promising in promoting lacrimal gland function in the treatment of tear deficient dry eye, such as mucolytics (bromhexine and ambroxol), cholinergic agents (carbachol, bethanecol, pilocarpine), or eledoisin (Calonge 2001). In general, topical drugs that increase cyclic nucleotide (cAMP or cGMP) levels can theoretically increase tear secretion (Gilbard, Rossi et al. 1990). However, the clinical efficacy of this therapeutic strategy for dry eye remains undetermined so far.

Since the causes of dry eye are multifactorial, the therapy should improve the symptoms and signs of dry eye as well as address the underlying inflammatory pathophysiology of the disease. Topical corticosteroids have short-term efficacy for Sjögren's syndrome, however, the likelihood of steroid-related complications limits their long-term use (Marsh and Pflugfelder 1999). In severe blepharitis, especially in rosacea-associated meibomitis, oral tetracycline and its derivatives are beneficial because they inhibit the production of lipase and thus lower the level of free fat acids. Clinically it has been observed that this group of antibiotics reduces lid margin inflammation and enhances meibomian gland secretion (Dougherty, McCulley et al. 1991) (Frucht-Pery, Sagi et al. 1993). A cyclosporin based new formulation has recently become commercially available and for the first time provides the opportunity for long term immunosuppressive topical treatment of the inflammatory component of dry eye pathogenesis. However, from veterinary experience it is already known that not all forms of dry eye respond to treatment with topical cyclosporin and that its long term efficacy is often limited (Mendicute, Aranzasti et al. 1997; Sall, Stevenson et al. 2000).

Tear preservation can be achieved by occlusion of the lacrimal canaliculi with punctal plugs, such as collagen (temporary) and silicone (semi-permanent) (Murube and Murube 1996). They improve the objective signs and symptoms of some dry eye patients, but silicon plugs can be associated with problems such as irritation, extrusion, migration along the canniculus, canaliculitis and dacrocystitis (Rumelt, Remulla et al. 1997). Moist chamber spectacles and wet gauze eye masks are effective in reducing tear evaporation from the ocular surface (Kurihashi 1994; Farris, Kornfield et al. 2000), but seriously limit the patients' vision and hence the quality of life.

1.2.4 Drawbacks of current artificial tears

Artificial tear substitutes have been used for treating dry eye syndromes for decades and succeeded in enhancing the comfort of patients. They are currently the main therapy for dry eye and likely to remain the mainstay. However, the currently used artificial tears have obvious limitations. The main purpose of using artificial tears is to lubricate the ocular surface. Artificial tear substitutes so far do not replace the nutrient function of natural tears (Tiffany 1994; McCulley and Shine 2001; Miano, Mazzone et al. 2002). Another important drawback is the fact that, in order to ensure a long shelf-life, they often contain

preservatives, stabilizers, and other additives, which potentially induce toxic or allergic reactions (Murube, Murube et al. 1998) (Tripathi and Tripathi 1989; Tripathi, Tripathi et al. 1992) (De Saint Jean, Brignole et al. 1999). Furthermore, artificial tears are applied intermittently, rather than continuously as are natural tears. One of the major problems encountered with solutions in eye is the rapid and extensive elimination of drugs from the precorneal lacrimal fluid reservoir by drainage or lacrimation. Consequently, the ocular residence time of most eye drops is limited to a few minutes and the overall absorption of a topically applied drug is limited to 1 to 10% of the applied active agent (Mishima 1981). To overcome this problem, formulations can either be substituted with mucoadhesive components or the lacrimal drainage system can be blocked to increase their contact time with the ocular surface and thus to extend symptomatic control (Snibson, Greaves et al. 1992) (Oechsner and Keipert 1999).

1.3 THE NEW CONCEPT OF A NUTRIENT OCULAR SURFACE LUBRICANT

In order to overcome the above drawbacks of artificial tears, a new artificial substitute capable of providing lubrication and nutrition in one with extended ocular surface residence time needs to be developed. The following list provides the key elements which need to be considered when formulating an artificial tear substitute (Sibley 1994):

- *Selection of active ingredients*
- *Decision on salt composition / osmolarity*
- *Selection of viscosity agents*
- *Choice of pH / buffering agents / buffering capacity*
- *Inclusion of other ingredients*
- *Exclusion or inclusion of preservatives*
- *Avoidance of toxicity to the ocular surface*

It is generally accepted that the pH and osmolarity of a tear substitute should be in the range of normal tears, in order to allow full recovery of epithelial barrier function (Thomas, Szeto et al. 1997). It is also known that viscosity and surface tension (ST) as well as mucoadhesive ability to the ocular surface play an important role in the maintenance of an intact tear film and in achieving an adequate residence time of tear substitutes (Lemp and Holly 1972; Lemp and Szymanski 1975). Drug related toxicity should be avoided,

since tear substitutes are instilled many times per day and might otherwise induce further deterioration of the ocular surface.

In this study, I have assessed the nutrient and lubricant properties of a defined cell culture medium supplemented with various viscosity modifying substances.

1.3.1 Basic medium

A defined cell culture medium called Defined Keratinocyte Serum Free Medium (**DKSFM**) was chosen as the basic solution for the formulation. This is an extensively characterised serum free cell culture. DKSFM is a growth enhancing medium especially designed for keratinocytes in the laboratory situation. It has only recently become commercially available and was so far not evaluated for use in humans. The main rationale is that the culture medium may create an optimal microenvironment for the ocular surface, since it provides nutrient constituents required for cell growth and proliferation. Serum free medium is designed for universal use in culturing mammalian cell lines and it has three basic advantages over any serum containing medium. These include:

1) A simplified and better defined composition,
2) A reduced risk of bacterial contamination,
3) Elimination of potentially infectious components.

The use of serum free medium has not only become routine in many laboratories for the culture of a wide variety of cell types, but has also been reported as a treatment for chronic ulcers *in vivo* (Lindenbaum, Har Shai et al. 2001).

The most outstanding feature of DKSFM is that its composition is characterised and the concentration of each component precisely defined. Besides trace elements, amino acids and vitamins it contains hormones such as insulin, hydrocortisone and triiodothyronine, as well as recombinant growth factors such as EGF and fibroblast growth factor (FGF). In quality control testing the composition of the medium was found to be stable for a minimum of 90 days. The manufacturer could show that DKSFM demonstrates superior primary cell growth while maintaining morphology and physiological marker. It has been used successfully to grow primary human corneal epithelial cells in the laboratory (Geerling, Daniels et al. 2001).

In a pilot *in vitro* toxicity study it was used as the "gold standard" against which serum drops and other tear substitutes were compared (Geerling, Daniels et al. 2001). From this it is known that this defined medium is capable of maintaining cell surface morphology, cell membrane integrity and intracellular level of ATP of cultured epithelial cells *in vitro* better than any other tear substitute tested, including serum eyedrops. In addition it has been tested at a collaborating institution (Moorfields Eye Hospital / London) in an uncontrolled clinical pilot study in dry eyes (n = 10) with 8 topical applications a day for 4 weeks. This pilot study strongly suggested a beneficial effect of DKSFM and did not reveal any side effects. DKSFM was thus chosen as the basic agent to formulate a nutrient tear substitute and supplemented it with various agents to further enhance its biophysical or biochemical properties.

1.3.2 Lubricating supplements

An important parameter of tear substitutes is the time that its active components are retained on the ocular surface. Any liquid applied to the ocular surface can be lost by spillage or – if not occluded for therapeutic reasons – by drainage through the naso-lacrimal apparatus. The normal tear volume is 7 µL and when this exceeds 7-10 µL, i.e. due to reflex tearing, naso-lacrimal drainage takes place although the fornices and palpebral aperture can accommodate up to 30 µl without spillage. Since the estimated volume of a drop ranges from 34 to 63 µL (German, Hurst et al. 1999), partial loss of a topically applied medication should always be expected. This can be through drainage, evaporation or conjunctival blood vessels, which remove any superficially adsorbed components. Natural tear production is virtually constant, but artificial tears are delivered intermittently. Therefore, the artificial tears must last longer than normal tears in the lacrimal basin. Increasing the frequency of application and incorporating viscous agents in tear substitutes are the main methods to prolong the residence time and to improve symptomatic relief in dry eyes. Viscosity is a key element of an artificial tear product, which can achieve extended ocular surface residence time. However, DKSFM was not designed for use in dry eyes and therefore its lubricating properties have not been optimised so far.

I have supplemented DKSFM with three currently used viscous agents. These were:

1) Hydroxypropylmethylcellulose (hypromellose = HPMC) or

2) Carbopol 980 (carbomer = polyacrylic acid = PAA) or

3) Sodium hyaluronate (SH; hyaluronate = hyaluronic acid = HA).

In the subsequent text I refer to these as either **DKSFM-variations** or more specifically to

1) DKSFM-HPMC

2) DKSFM-Carbopol

3) DKSFM-SH

Preservatives were not added to any of these DKSFM-variations.

HPMC is synthesized from methylcellulose as a raw wood pulp product. It consists of long chains of glucose molecules with replacement of the hydrogen of the hydroxyl groups by methoxy and hydroxypropyl side-chains. It belongs to the family of substituted cellulose ethers and is often used as a first line treatment for dry eyes or ocular surface disease (Toda, Shinozaki et al. 1996). Although its viscosity is relative lower than carbomer, it was shown to have superior mucoadhesive properties (Chary, Vani et al. 1999).

Carbopol, a member of the carbomer family, is crosslinked, polymerized carboxyvinylic acid with high molecular weight. The advantages of a carbomer gel-based artificial tears over other formulations are an excellent adhesive behaviour, higher retention time, and better efficacy in reducing dry eye symptoms (Marquardt and Christ 1986) (al-Mansouri, Tabbara et al. 1994) (Marner, Mooller et al. 1996). It is assumed that molecules of carbomer are highly entangled and have many cross-links which result in high molecular attractions and a high viscosity (Islam, Rodriguez-Hornedo et al. 2004). However, the disadvantage of carbomer is that a highly viscous gel is formed at the concentration of 0.2%, which can irritate the eye and blur vision (Marner, Mooller et al. 1996).

SH is a natural component of tears synthesised by corneal epithelial cells. It is a large polysaccharide molecule found in nearly all connective tissues. Hyaluronate is a linear polymer composed of long chains of repeating disaccharide units of N-acetylglucosamine and glucuronic acid. It is widely used in ophthalmic surgery because of its viscosity and pseudoplasticity. Hyaluronate is also known to promote corneal epithelial wound healing (Inoue and Katakami 1993) (Yokoi, Komuro et al. 1997).

1.3.3 Nutrient supplements

Two additional nutrient substances, allantoin and fibronectin, were also tested in addition in order to further enhance the nutrient ability of DKSFM. These additional DKSFM-variations are termed:

4) DKSFM-allantoin

5) DKSFM-fibronectin

Allantoin is a uric acid derivative that has astringent and keratolytic properties. It is present in multi-ingredient preparations intended for various skin disorders. It was found to stimulate cell proliferation and be helpful in healing skin wounds and ulcers (Margraf and Covey 1977). Silver-zinc-allantoin powder has successfully been used in the treatment of severe thermal skin burns (Klippel, Margraf et al. 1977) (Sheker, Black et al. 1972). It also improves proliferation of Schwann cells in posttraumatic neuropathies (Loots, Loots et al. 1979).

Fibronectin is a large multidomain glycoprotein present in connective tissue, on cell surfaces, in plasma and other bodily fluids in soluble form and in extracellular matrices in insoluble form. It serves as a temporary matrix over which corneal epithelial cells migrate during wound healing and has been found to be chemotactic and haptotactic. Fibronectin has been used *in vitro* and *in vivo* in the range of concentrations from 0.0001% to 0.35% (Phan, Foster et al. 1989) (Gordon, Johnson et al. 1995) (Nakamura, Sato et al. 1997) (Phan, Foster et al. 1987). It stimulates the attachment of corneal epithelial cells (Er and Uzmez 1998) and thus plays an essential role in wound healing (Spigelman, Deutsch et al. 1987; Nakamura, Sato et al. 1997). It has been shown that topical autologous plasma fibronectin is beneficial in persistent corneal epithelial defects (Nishida 1983). However, due to its limited stability and efficacy when used as a single active component, and its time- and labour intensive producing way by means of purification by eluting with urea from plasma, fibronectin has not entered the portfolio of routine clinical management for ocular surface disease.

1.4 PURPOSE OF THE INVESTIGATION AND SCIENTIFIC QUESTIONS

The goal of this project was to produce a new nutrient tear substitute with improved lubricant character. I therefore attempted to optimise the biophysical properties, i.e. the viscosity and surface tension (ST) as well as the epitheliotrophic activity of a cell culture medium by supplementing it with various agents. In order to avoid *in vitro* toxicity and to enable later use in a clinical trial the pH, osmolarity and the stability of these DKSFM-variations also had to be assessed and controlled.

Primary questions were:

(1) What are the biochemical and biophysical properties of DKSFM and the DKSFM-variations produced?
(2) Does supplementation of DKSFM with pharmacy grade single compounds or ready made pharmaceutical tear drops reduce or improve its nutrient properties?

Answering these questions should ultimately allow concluding which supplement and at what concentration will be favourable for a clinical trial.

By comparing two conjunctival and two corneal epithelial cell lines as well as two different endpoint assays to assess toxicity (luminescence based quantification of intracellular ATP versus penetration and/or turnover of a combination of two fluorescent dyes) I also aimed to answer a number of ***secondary research questions***:

(3) Which of the two quantitative assays detects cytotoxicity at the lower concentration of the test substances?
(4) Do various ocular epithelial cell lines show a similar response, in terms of support of proliferation upon exposure to the substances tested and how does this response compare to primary epithelial cells?

2 MATERIALS AND METHODS

2.1 MATERIALS

DKSFM was supplemented with pure powders of HPMC, Carpobol or SH to adjust its biophysical parameters and allantoin or fibronectin to improve its epitheliotrophic properties. The supplements used were all single pharmacy grade compounds, which had to be dissolved in the DKSFM. In order to exclude any toxicity from dissolving the single compounds I also tested ready made unpreserved eyedrops / ophthalmic products containing the same active viscosity agents as controls. These were Hypromellose® as control for HPMC, Thilo-Tears® SE as control for carbopol and Vislube® or Healon® GV for SH. These DKSFM-variations are referred to as:

DKSFM-Hypromellose®

DKSFM-ThiloTears®

DKSFM-Vislube®

DKSFM-Healon®

The compositions of DKSFM and the control solutions are given in the appendix Tab.1. The sources of the materials were:

Basic solution:

DKSFM and Growth Supplement (GS) (Gibco-BRL, Life Technologies, Grand Island, NY, USA)

Supplements:

HPMC (Dow Chemical Co., Midland, MI, USA)

Carpobol 980 (Noveon, Inc., Cleveland, OH, USA)

SH (Fluka, Sigma-Aldrich, Chemical Co., Ltd., Poole, UK)

Allantoin (Sigma-Aldrich, Chemical Co., Ltd., Poole, UK)

Fibronectin (Sigma-Aldrich, Chemical Co., Ltd., Poole, UK)

Controls:

Hypromellose® (0.3% HPMC, Pharmacy of Moorfields Eye Hospital, London, UK)

Thilo-Tears® SE (0.3% Carbopol 974P, Alcon Phama GmbH, Freiburg, Germany)

Vislube® (0.18% SH, TRB CHEMEDICA AG, Munich, Germany)

Healon® GV (1.4% SH, Pharmacia, Uppsala, Sweden)

2.2 PREPARATIONS OF TEST SOLUTIONS

All variations of DKSFM and control solutions were prepared under sterile conditions in a laminar air flow hood. The basic medium solution was prepared from the product package of DKSFM containing 500 mL liquid basal medium and a 1 mL frozen GS. The frozen GS was thawed in a 37° C water bath, mixed by gentle pipetting of the solution and the entire contents of the vial was aseptically transferred to the bottle of DKSFM basal medium. The vial was rinsed with medium and this volume added to the bottle of defined medium. The bottle of medium was gently swirled to ensure complete mixing. The term **"DKSFM"** refers to the complete medium containing the basic medium plus the growth factor supplement.

All single compound supplements - except for fibronectin - were dissolved in DKSFM at concentrations of

0.4% **0.2%** **0.1%** **0.05%** **0.025%** **0.0125%**

These figures – with exemption of the top concentration - refer to the concentration of the supplemented agent in DKSFM, regardless of its source, be it from a single compound source or a more complex ready made eyedrop. With one exemption none of the DKSFM-supplement variations contained antibiotics, although antibiotics are commonly used in cell culture. The reason for this was that I intended to test only solutions that could be used in a clinical setting and antibiotics can induce toxicity (Li, Kuentzel et al. 1977; Yust, Frisch et al. 1981). When stored for up to 3 months at 4°C none of these solutions showed apparent microbial contamination, except for DKSFM-SH, which became contaminated with bacteria within one month after manufacture. It is well known that bacteria bind easily to the glucose molecule in hyaluronate, I therefore added 100 µg/mL streptomycin, and 100 IU/mL penicillin, which have been tested on the HCE-T cell line in the Laboratory for Experimental Ophthalmology at the University of Lübeck and were found not to be toxic at the above concentrations (Verbal communication with K. Kasper). Cooper also showed that culture medium with 10,000 U/mL penicillin and 10,000 mg/L streptomycin had no effect on keratinocyte growth compared to culture medium without antibiotics (Cooper, Boyce et al. 1990).

The top concentration of 0.4% for the DKSFM-variations was chosen slightly above the concentration of the corresponding viscous agent in the commercially available tear substitutes used as control sources, which have a maximum concentration of

0.3% of HPMC in **Hypromellose®**
0.3% of Carbopol in **Thilo-Tears® SE** and
0.18% of SH in **Vislube®**

The top concentration of these commercial products in DKSFM were

0.2% for DKSFM-Hypromellose®
0.2% for DKSFM-Thilo-Tears® SE and
0.1% for DKSFM-Vislube®

The concentrations of DKSFM-fibronectin were chosen differently in view of the high costs for its purchase from a commercial supplier. Since Nakamura found in a rat model that 0.0001 to 0.1% of fibronectin improve corneal epithelial wound healing in a dose-dependent manner and 0.1% is significantly more effective than PBS (Nakamura, Sato et al. 1997) I chose the latter as my top concentration in DKSFM.

The concentrations of **DKSFM-fibronectin** therefor were

0.1% **0.01%** **0.005%** **0,001%** **0.0001%**

Stock solutions of DKSFM were first prepared with HPMC, Carbopol, SH, allantoin to the concentration of 0.4%. Allantoin was easily dissolved by gentle swirling. Since the viscous agents require extended time to go into solution, and in order to prevent growth factor degradation during this time HPMC, SH and carbopol were added to the DKSFM in a 4°C cold room or cabinet and stirred until completely dissolved. Carbopol tends to form clumps of particles when haphazardly dispersed. In order to achieve complete dispersion, Carbopol had to be added slowly and carefully to the DKSFM while the mix was being stirred. To insure complete solution, the pH was measured and adjusted by 1N sodium hydroxide (NaOH) to 7.2 which is in the range of the tear level (7.2-7.6). The stirring was continued

for 24 h in a 4°C cold room. Fibronectin was dissolved in DKSFM to 0.1% and stirred in a cold room to complete.

The pH, osmolarity, viscosity and surface tension of DKSFM and all concentrations of DKSFM-variations were measured. The chemical properties were mainly determined to exclude an unphysiological pH or osmolality, which both can induce toxicity (Geerling, Daniels et al. 2001) (Xiao, Li et al. 2003). For the commercial tear substitutes only the original undiluted solution and for DKSFM-fibronectin only the top-concentration were measured. These measurements were repeated after 3 months of storage at 4°C for DKSFM-HPMC, -Carbopol, -SH and -allantoin at the top concentration to evaluate their stability. All the above solutions were tested for their effect on cell proliferation and viability, but only the two concentrations of each test solution that were found to have little or no toxicity were tested in the cell migration assay.

2.3 CONTROL OF CHEMICAL PROPERTIES

2.3.1 Control of pH

The pH of all the solutions was measured at room temperature by an Ion Selective Field Effect Transistor (ISFET) pH meter (pH Boy 501, CamLab Ltd, Cambridge, England). The pH meter has a measuring range of 2.0 to 12.0 with a reproducibility of ± 0.1 pH. Before the measurement, the pH meter was calibrated by pH 6.9 standard buffer solution. One drop of the test solutions was pipetted onto the ISFET electrode and readings were displayed on a digital screen.

2.3.2 Control of osmolarity

The osmolarity of all the solutions was measured with a Vapor Pressure Osmometer (model 5500, Wescor, Logan, Utah, USA) at room temperature. The instrument measures osmolarity of 10 µL samples over the range of 0 to 3200 mOsm/L. Prior to the measurement, the osmometer was calibrated with a standard solution of 290 mOsm/L osmolarity. A 6 mm filter paper discs were placed in a disk holder centred with a pair of forceps and a 10 µL standard solution was pipetted onto the centre of the paper disc

without producing air bubbles. The runs of the measurement were performed automatically after loading the samples and the readings were taken after a signal.

2.4 EVALUATION OF PHYSICAL PROPERTIES

2.4.1 Determination of surface tension

The measurements were performed in cooperation with John M. Tiffany at the Nuffield Laboratory of Ophthalmology/Oxford/UK. The surface tension was measured at room temperature using a capillary-micro-technique that was developed by Tiffany, Winter and Bliss (Tiffany, Winter et al. 1989) based on the method of Ferguson and Kennedy (Ferguson and Kennedy 1932). A 1µL of sample was collected in a 10 µL glass capillary tube. The tube was held horizontally and air pressure was applied until the pressure difference across the near-hemispherical meniscus at the open end was overcome and the meniscus appeared flat, as determined by reflection of light from the tube end to a viewing telescope. The applied pressure equalled 2T/r Pascals, where T was the surface tension in mN/m and r the radius of the tube in mm. The flattening pressure was measured by a sensitive electronic manometer (Air Instrument Resources Ltd, Chalgrove, Oxfordshire, UK: model MP6KMD).

2.4.2 Determination of viscosity

The viscosity of the DKSFM, the DKSFM-variations as well as the 3 commercial artificial tears was measured using the Contraves Low Shear 30 rheometer over the range of shear rates of 1.7 to 128.5 sec^{-1}. The measurements were performed in cooperation with John M. Tiffany at the Nuffield Laboratory of Ophthalmology/Oxford. The method was previously described by Tiffany (Tiffany 1991). The sample was held in the annular space between an outer cylindrical cup which can be rotated at a series of fixed speeds to five steps of constant shear rate, and an inner cylinder or bob suspended from a torsion wire. The restoring force which must be supplied electrically to prevent rotation of the bob due to viscous drag is converted by instrument calibration tables to values of apparent viscosity at constant rate of shear rate strain. An averaged value is used for the rate of shear since it is not constant across the annular gap by the method. The cup/bob combination I used

requires ca. 0.5 - 1.0 mL of sample solution. The range of the instrument is 4.1×10^{-2} - 1.9×10^{3} mPs.sec (or cP) or viscosity over 5 ranges of sensibility, and 1.7 - 128.5 sec^{-1} for shear rate in 30 steps. The temperature within the Couette cup was controlled at 37°C by circulation of water through the cup mounting since the temperature can alter viscosity values. Evaporative losses from the sample cup, which might change the sample concentration in the course of a run, were avoided by construction of a shield surrounding the hanging bob and the free surface of sample fluid in the cup, and controlling the humidity with water in a trough.

The sample was carefully filled into the cup by Eppendorf tips, avoiding air bubbles. The central position of the bob within the cup was checked before setting rotational speeds. Torque readings were taken at each rotational speed setting, starting from the lowest speed. The ranges were adjusted to have the readings below 100. A higher range was used when the readings were above 100 and the last lower 3 shear rates were measured again to ensure that there was no shear-degradation of the sample. The torque reading corresponding to each value of shear rate was converted to the apparent coefficient of viscosity using tables supplied by the instrument manufacturer.

2.5 CELL CULTURE MODELS

2.5.1 Cell cultures of human corneal and conjunctival epithelial cell lines

Four human cell lines of corneal and conjunctival epithelial origin were cultured at 5% CO_2, 37°C. The two human corneal epithelial cell lines are called HCE-T and CEPI-17-CL4 and the two conjunctival epithelial cell lines were Chang and IOBA-NHC. Each cell line was initially cultured with the medium described by its depositor / creator. In the subsequent text the equivalent medium for each cell line is referred to as "***original culture medium***".

HCE-T (human corneal epithelial cell line), stem from corneal epithelial cells (RCB1384, Riken Cell Bank, Ibaraki, Japan), immortalized by Sasaki et al with a SV-40-adenovirus vector and indicated the properties of normal corneal epithelial cells. It shows well-developed desmosome and abundant microvilli formation and expresses cornea-specific,

64-kD cytokeratin. The cells are able to form a multilayer if cultured at an air-liquid interface which indicates that these cells retain the programmed gene necessary for differentiation (Araki-Sasaki, Ohashi et al. 1995). I cultured HCE-T cells with 1:1 Ham's F12 (Biochrom AG, Berlin, Germany) and MEM (Gibco-BRL, Life Technologies, Grand Island, NY) supplemented with 25mM hepes puffer, 5% fetal bovine serum (FBS), 10 ng/mL EGF, 5 µg/mL insulin, 0.1 µg/mL cholera toxin and 100 IU/mL penicillin, 100 µg/mL streptomycin.

CEPI-17-CL4, human corneal epithelial cells (Nestlé Research Center, Lausanne, Switzerland), were also immortalized with SV40-T retroviaral vector. Sharif et al have used the cells successfully to study functional and pharmacologic responses to key inflammatory agents such as bradykinin, histamine, and platelet-activating factor (Sharif, Wiernas et al. 1998). They were shown to be truly immortal and to express an extensive array of cytokines (interleukin [IL]-1α, IL-1β, IL-6 and IL-8, tumor necrosis factor-α, and IL-ra), growth factors (transforming growth factor [TGF]- α, EGF, EGF receptor, TGF-β_1, TGF-β_2, and platelet-derived growth factor [PDGF]-β) and cytochrome P450 enzymes (A1, 2C, 2E1, and 3A5) that resemble the original tissue (Offord, Sharif et al. 1999). I cultured the cells with Keratinocyte Growth Medium (KGF, Biofluids, Rockville, MD, USA) containing 30 µg/mL Bovine Pituitary Extract (BPE), 0.5 µg/mL hydrocortisone, 5 µg/mL insulin, 10 µg/mL transferrin, 10 ng/mL EGF, 0.15 mM calcium chloride, 0.05 µg/mL amphotericin B and 50 µg/mL gentamincin.

Chang, Wong-Kilbourne derivative of Chang conjunctival cells, (CCL-20.2, Clone 1-5c-4, American Type Culture Collection [ATCC], Rockville, MD, USA) are continuous untransfected epithelial cells derived from human conjunctiva (Chang 1954), which are known to have become contaminated at some stage by HeLa cells (Lavappa, Macy et al. 1976). The cell line has been used to study the expression of inflammation-related markers such as CD40 antigen (Bourcier, De Saint-Jean et al. 2000) or apoptosis (De Saint Jean, Brignole et al. 1999; De Saint Jean, Debbasch et al. 2000). The cells were cultured in Eagle's minimal essential medium (Gibco-BRL) supplemented with 5% FBS, 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 IU/mL penicillin.

IOBA-NHC, normal human conjunctiva epithelial cell line (IOBA-University of Valladolid, Valladolid, Spain), is the only nontransfected, spontaneously immortalized cell

line derived from normal human conjunctival epithelium. This cell line has been reported to retain morphologic and functional conjunctival epithelial characteristics in vitro. Cytokines such as CK-3 and CK-7 and several lectins including GalNAc, GluNAc and mannose were immunodetected. Ultrastructural details such as desmosomes, microvilli on the cell surface and intermediate filaments in cytosol were observed by electron microscope. (Diebold, Calonge et al. 2003) I cultured the cells with 1:1 DMEM/F12 (Gibco-BRL) supplemented with 1 µg/mL insulin, 2 ng/mL EGF, 0.1 µg/mL cholera toxin, 5 µg/mL hydrocortisone, 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B.

All the medium supplements were purchased from Sigma-Aldrich Co., Ltd. except that FBS and all antibodies were from Gibco-BRL. Cells were cultured in 75 cm^2 plastic flasks (Greiner bio-one GmbH, Frickenhausen, Germany) and the media were changed every 2 or 3 days. After confluence, the cells were subcultured with 5 mg/mL trypsin-EDTA (Biochrom AG) and 5 mg/mL soybean trypsin inhibitor (Sigma-Aldrich Co., Ltd.) and split at a ratio of 1:4 or 1:6 according to the cell number. About 10^6 cells were seeded in a 75 cm^2 plastic flask.

2.5.2 Cell cultures of primary rabbit corneal epithelial (RCE) cells

One male adult New Zealand rabbit was provided by the animal centre of the University of Lübeck. The animal was handled according to the guidelines described in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbit was killed by intravenous injection of sodium pentobarbital solution (100 mg/kg) and the globes were enucleated. Two corneas were excised including the corneal rim and washed three times with 100 µg/mL streptomycin and 100 IU/mL penicillin. Each cornea was cut into 4 sections and incubated at 37°C for 2 h in 2mg/mL Dispase II (Boehringer-Mannheim, Mannheim, Germany) dissolved in DMEM containing 10% serum. The epithelium was stripped off with gentle scraping from the limbus to the centre into the well of a 6-well-plate containing 3mL PBS. The solution was pipetted to disperse cells and centrifuged at 100g for 5 minutes. PBS was carefully removed and the cells were suspended in 5mL Keratinocyte-SFM (Gibco-BRL) supplemented with 5 ng/mL EGF, 50 µg/mL BPE, and 100 IU/mL penicillin and 100 µg/mL streptomycin and seeded in a 25

cm^2 cell culture flask. The cells were cultured at 37°C with 5% CO_2 until confluent and expanded by using routine cell cultured technique.

2.6 IMMUNOHISTOCHEMISTRY

The expression of epithelial cell specific markers, cytokeratins (CK3 and CK19), was investigated for rabbit corneal epithelial cells and cell lines using the Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) method. CK3 and CK19 as demonstrated by Elder et al (Elder, Hiscott et al. 1997) are able to discriminate between corneal and conjunctival epithelia: CK3 stains all layers of normal human corneal epithelium but does not stain the conjunctival cells, whereas CK19 stains the conjunctival but not the corneal epithelium.

Ten thousands cells were seeded on plastic cell culture inserts (Thermanox, Nalge Nunc Int'l, Rochester, NY, USA) in a 24-well-plate and incubated with culture medium for 24 h. The cells were fixed with formalin, washed with TBS, and incubated in blocking buffer (SNS) for 30 minutes at room temperature and before they were exposed to primary antibodies, 1: 75 monoclonal mouse anti-CK3 (ICN Biomedical, Inc. Irvine, CA, USA) and 1: 75 monoclonal mouse anti-CK19 (DAKO, Dianostika GmbH, Humburg, Germany) overnight. After washing, the cells were incubated in 1: 50 rabbit anti-mouse IgG (DAKO) for 20 minutes at room temperature. The cells were then washed and incubated in 1: 50 APAAP-Complex (Dianova GmbH, Hamburg, Germany) for 30 minutes at room temperature, stained with neufuchsin and mounted on slides. Human corneal and conjunctival tissue sections stained with both CK3 and CK19 were used as positive and negative controls with the same procedures as above, except that negative controls were incubated without primary antibodies. The slides were observed under the light microscope and photographed.

2.7 ENDPOINT ASSAYS

2.7.1 Viability/Toxicity: Calcein AM/EthD-1 assay

The Calcein AM/EthD-1 assay can simultaneously determine live and dead cells with two probes that measure two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity. The nonfluoresecent calcein-acetoxymethyl (Calcein-AM) is converted into green fluorescent calcein by intracellular esterase as an indicator of a viable cell metabolism. The second dye, Ethidium-homodimer (EthD-1), is usually excluded by viable cells. However, it permeates damaged cell membranes and binds to nucleic acids thus resulting in a red fluorescence. Dead or dying cells therefore stain red while viable cells stain green. Toxicity was defined as an increase in cell membrane permeability and reduction of esterase activity.

The experiments were performed in triplicates with one human corneal epithelial cell line (HCE-T) and one conjunctival epithelial cell line (IOBA-NHC). DKSFM-HPMC, -Carbopol, -SH, and -allantoin as well as the commercial tear substitutes diluted in DKSFM at the concentrations described above were tested. DKSFM and 1% benzalkonium chloride (BAC, Haltermann Ltd, Workington, UK) were used as positive and negative control.

Ten thousands cells were seeded per well in black 96 well plates with flat clear bottom (Costar, Corning Inc. Corning, NY, USA). After complete confluence, cells were exposed to the test substances for 24 and 72 h. After washing with PBS twice, the cells were incubated with 150 µL 4 µM EthD-1/2 µM calcein AM (Molecular Probes, Leiden, The Netherlands) diluted in PBS for 30 min at room temperature. The fluorescence of cells was measured with a microplate reader (FLUOstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany) at excitation/emission of 485/520 nm and 520/645 nm wavelength. The green fluorescence intensity detected at 485/520 nm is proportional to the number of live cells and red fluorescence intensity detected at 520/645 nm is proportional to the number of dead cells. The percentage of green fluorescence, i.e. the cell viability (CV) for each drug and test situation was calculated

$$\frac{\text{Test - MI}}{\text{MO - MI}} \times 100 = \text{ \% CV},$$

where MO is mean green fluorescence of control cell cultures without inhibition (incubated with DKSFM), MI is mean fluorescence of control cell cultures with maximum inhibition (incubated with 1% BAC), and Test is mean fluorescence of cell cultures for triplicate test solutions.

The plate was then inverted carefully and the cells were viewed and photographed under the fluorescence microscope (Axioskop, Microskop-Kamera MC 100; Carl Zeiss Jana GmbH, Jena, Germany) at 490 nm excitation and 520nm emission wavelength. Dead or dying cells were stained red while viable cells were green.

2.7.2 Proliferation: ATP assay

The luminescence based ATP assay is proven to be a very sensitive tool to quantify cell proliferation (Crouch, Kozlowski et al. 1993) (Petty, Sutherland et al. 1995). The ATP assay system is based on the production of light caused by the reaction of ATP with added Luciferase and D-Luciferin. The amount of ATP in cells correlates with cell viability. Within minutes after a loss of membrane integrity, cells lose the ability to synthesize ATP, and endogenous ATPases destroy any remaining ATP, thus the levels of ATP fall precipitously and rapidly.

All ATP cell culture experiments were performed in triplicates and at least repeated once. The series of doubling dilutions or time points of incubation with the test substances applications was used to establish a dose or time response curve. The index "area under the curve" can be used to compare the cellular responses to the different test solutions. DKSFM-HPMC, -Carbopol, -SH, -allantoin, and -fibronectin as well as control solutions at all the test concentrations were tested, with DKSFM as positive control and 1% BAC as negative control. Time response curves were established using an incubation time of 24, 48, 72, 96 and 144 h (Geerling, Daniels et al. 2001). The four human epithelial cell lines were used in parallel for all the measurements. Primary RCE cells were used in 24 h-dose response experiments only.

Per well 3,000 cells were seeded in 96 well culture plates and cultured until 30% confluence with the original culture media as described above for the cell lines. The medium was then changed to a so called "***non-growth supporting medium***" containing

DKSFM with 1% serum albumin and 100 IU/mL penicillin, 100 µg/mL streptomycin and the cells were cultured for further 24 h. The cells were washed with PBS once and exposed to 200 µL test substances for various time points. Following this the test substances were removed and cells were washed with PBS once before 100 µL PBS and 50 µL of mammalian cell lysis solution were added to each well. The plate was shaken for 20 min in an orbital shaker at 200 rpm at room temperature. 50 µL cell extract was transferred to a white 96 half area well plate and 25 µL substrate (Luciferase/Luciferin) solution was added to the wells. The plate was shaken for 5 min in an orbital shaker at 200 rpm and dark adapted for 10 min before the luminescence was measured with a microplate reader. The luminescence intensity is proportional to the amount of ATP of cells. Since ATP is the marker of viability and present in all metabolically active cells, the percentage of cell growth (CG) for each drug and test situation was calculated with the same formula as for the fluorescent life dead assay:

$$\frac{\text{Test - MI}}{\text{MO - MI}} \times 100 = \%\ \text{CG},$$

where MO is mean counts for no inhibition control (DKSFM) cultures, MI is mean counts for maximum inhibition control (1% BAC) cultures, and Test is mean counts for triplicate test solutions.

2.7.3 Migration: Colony dispersion assay

The colony dispersion assay is used for assessing the effects of pharmacological compounds on the motility of cells and for assaying the migratory capacity of various different cell lines in parallel. This assay was performed by observing cell migration on collagen I-coated plates after prohibiting cell proliferation. Cell migration plays a central role in wound healing, so it provides important information for composing nutrient DKSFM (Lu, Reinach et al. 2001).

This assay is used to measure cell migration, which is correlated with cell colony dispersion area. To reduce the number of experiments and since the 4 cell lines were found in the proliferation assay to show very similar responses on exposure to the test substances I decided to limit the colony dispersion assay to, although performed again in triplicates, to one corneal (CEPI-17-CL4) and one conjunctival epithelial cell line (Chang).

In every well 20,000 cells were seeded and cultured to confluence with original culture medium in triplicate cloning rings (flexiPERM micro 12, Vivascience, Sartorius AG, Goettingen, Germany) on 6 well plates pre-coated with 0.01% acid-extracted rat tail collagen I (Sigma-Aldrich, Co., Ltd.). The cells were cultured for a further 24 h in the presence of 200 µM hydroxyurea (Sigma-Aldrich, Co., Ltd.) to induce growth arrest (Pilcher, Dumin et al. 1997) and then starved for 24 h with the non-growth supporting medium. After removal of the rings, the cells were thoroughly washed with PBS and incubated with test substances for 24, 48, 72, 96 and 144 h.

Based on the ATP- and the Calcein AM/EthD-1 assay, two concentrations of each supplement (except for fibronectin and Healon®) with lower cytotoxicity were chosen and tested in a colony dispersion assay for the above mentioned time points. These concentrations were for

DKSFM-HPMC	0.2% and 0.4%
DKSFM-Carbopol	0.05% and 0.1%
DKSFM-SH	0.05% and 0.1%
DKSFM-allantoin	0.025% and 0.05%.

In order to limit the use of the more expensive supplements, the effect of DKSFM-fibronectin and DKSFM-Healon® on migration was evaluated in dose-response experiments at 72 h only:

DKSFM-fibronectin	0.0001%, 0,001%, 0.005%, 0.01%, 0.1%
DKSFM-Healon®	0.0125%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4%

Following exposure the cells were washed with PBS three times, fixed with 90% (vol/vol) methanol and stained with Mayer's hematoxylin. Dispersion areas were photographed with a digital camera (Sony Corporation, Japan) under standard conditions and the size of colony area was measured in pixels with an image-analysis software (Uthesca Image Tool, Version 2.00) and compared with the area of the zero h. (Daniels, Limb et al. 2003)

2.8 DATA EVALUATION AND STATISTICAL METHODS

Statistical analysis was performed with SPSS for windows (version 11.0.1). The Wilcoxon-test was used for viscosity measurements and the analysis of variance (ANOVA) test for the ATP assay. Linear-regression-test was employed for pseudoplasticity and colony-dispersion-assay. Chi-square test was used for comparison of ATP assay and Calcein AM/EthD-1 assay. $P \leq 0.05$ was considered statistically significant.

3 RESULTS

3.1 BIOCHEMICAL AND BIOPHYSICAL PROPERTIES OF DKSFM-VARIATIONS

The pH, osmolarity, ST and viscosity were measured for DKSFM, DKSFM-HPMC, -Carbopol, -SH and -allantoin at concentrations of 0.0125%, 0.025%, 0.05%, 0.1%, 0.2% and 0.4%, original commercial tear substitutes as well as DKSFM-fibronectin at concentrations of 0.0001%, 0.001%, 0.005%, 0.01% and 0.1%. The measurements for pH, osmolarity and ST are given in Table 2 and for viscosity in Table 3 of the appendix.

3.1.1 pH

The pH of DKSFM was 7.2 and this was not altered by supplementing it with HPMC or SH. DKSFM-allantoin and -fibronectin at all the concentrations slightly increased the pH to 7.3. The pH of all commercial solutions were near the physiological level with Hypromellose® at 7.8, of Thilo-Tears® at 7.1 and Vislube® at 6.9. Carbopol was the only substance changing the pH of DKSFM significantly. After slowly adding Carbopol powder to the DKSFM to the concentration of 0.4% and stirring this for a few minutes, the pH of the solution dropped to about 3.0 and had to be neutralised to 7.2. Since it was found that the Carbopol was not completely dissolved at this time, it was kept stirring for 24 h when it was found to be dissolved completely, though the pH of the solution had dropped to about 6.0 and had to be adjusted again to 7.2. Following this serial dilutions down to 0.0125% DKSFM-Carbopol were prepared. The pH of all the solutions was remeasured after serial dilution and found to be stable at pH 7.2.

3.1.2 Osmolarity

The osmolarity of DKSFM was 278 mOsm/L and that of DKSFM-HPMC, -Carbopol, -SH, -Healon® and -allantoin ranged from 276 mOsm/L (0.025% DKSFM-Carbopol) to 308 mOsm/L (0.4% DKSFM-allantoin). The osmolarity of commercial Hypromellose® was 302 mOsm/L, Thilo-Tears® was 329 mOsm/L and Vislube® was 158 mOsm/L. The osmolarity of DKSFM-fibronectin rose to 569 mOsm/L at the concentration of 0.1%, but

dropped to 329, 340, 299 and 299 mOsm/L at 0.01%, 0.005, 0.001% and 0.0001% respectively.

3.1.3 Surface tension

The ST of all measurements were close to that of water (72 mN/m) and DKSFM (70.9 mN/m), except for DKSFM-HPMC and commercial Hypromellose® which were obviously lower at around 50 mN/m (Table 2 of the appendix).

3.1.4 Viscosity

Fig. 1 shows the viscosity values (mPa.sec) for DKSFM, DKSFM-HPMC, -Carbopol, -SH, -allantoin and -fibronectin at test concentrations as well as original commercial artificial tears, over the range of 1.7 - 128.5 s^{-1} shear rate at 37°C. The viscosity of normal human tears is 5 mPa.sec at a shear rate of 2 s^{-1} (Tiffany 1991). The viscosity of DKSFM was around 0.75 mPa.sec at all the shear rates examined and that of DKSFM-HPMC, -Carbopol and -SH at all concentrations was significantly higher than DKSFM alone (P = 0.001). 0.1% DKSFM-fibronectin had also a higher viscosity than DKSFM (P = 0.001), but the values of DKSFM-allantoin at all the concentrations was close to the original DKSFM (P = 0.668). Higher concentrations of DKSFM-Carbopol and -SH were more viscous than less concentrated solutions (P = 0.001). For DKSFM-HPMC, the viscosity at 0.2% was higher than at 0.1% (P = 0.001) and 0.025% higher than 0.0125% (P = 0.008), but there was no significant difference between 0.4% and 0.2%.

The viscosity of commercial Hypromellose®, containing 0.3% HPMC, was lower than 0.4% DKSFM-HPMC, but higher than 0.2% DKSFM-HPMC (P = 0.001). The viscosity of Vislube®, containing 0.18% SH, was lower than 0.4% DKSFM-SH and higher than 0.1% DKSFM-SH (P = 0.001), but not different from 0.2 % DKSFM-SH. The viscosity of Thilo-Tears® was not detectable since it was beyond the range of the viscometer.

Non-Newtonian rheology is indicated by higher viscosity at lower shear rate and lower viscosity at higher shear-rate. The non-Newtonian property was investigated for all the formulations with the linear regression test, which analyzes the relationship between two variables, viscosity and shear rate. DKSFM-HPMC, -Carbopol and -SH at concentrations

of 0.4% and 0.2% as well as commercial Hypromellose® and Vislube® showed non-Newtonian behaviour. However, the non-Newtonian property for 0.2% DKSFM-Carbopol and 0.4% and 0.2% DKSFM-SH was not shown apparently from the graphs. Other formulations were Newtonian.

1a

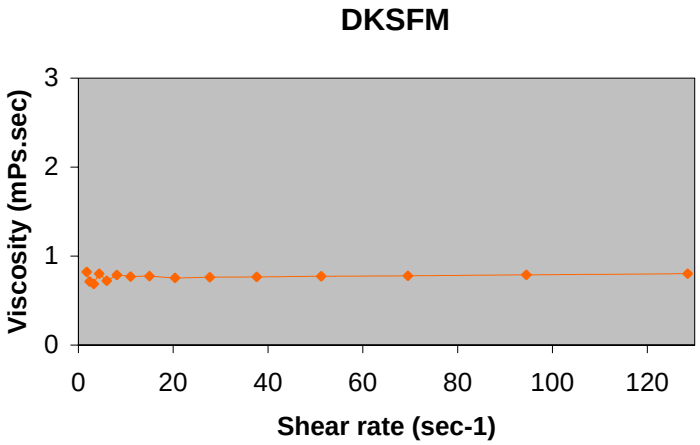


Fig. 1a: Viscosity of DKSFM was around 0.75 mPa.sec at all the shear rates examined.

1b

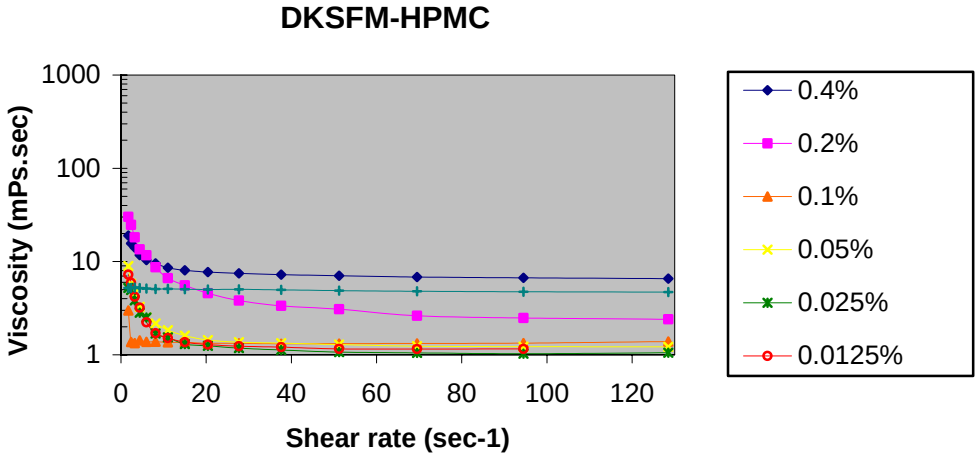


Fig. 1b: Viscosity of DKSFM-HPMC and commercial Hypromellose®. They all are more viscous than DKSFM. 0.2% and 0.4% DKSFM-HPMC have non-Newtonian property.

1c

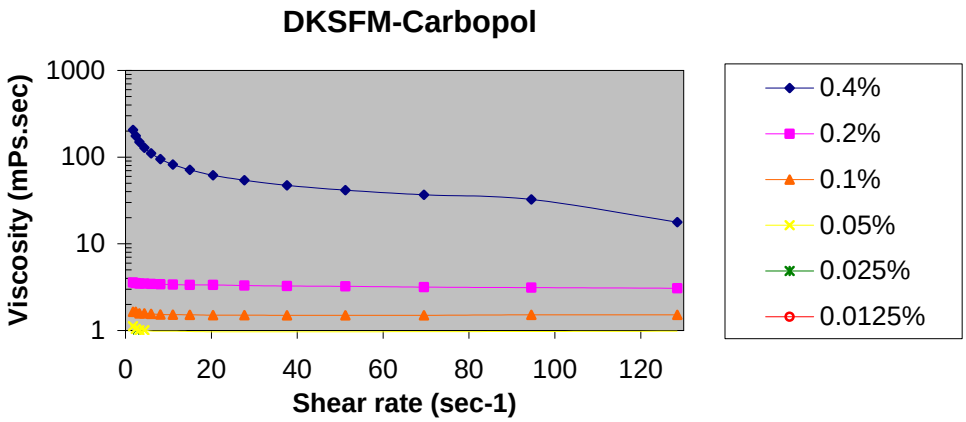


Fig. 1c: The viscosities of DKSFM-Carbopol at all the concentrations are significantly higher than DKSFM and it shows concentration-dependent. 0.2% and 0.4% DKSFM-Carbopol have non-Newtonian property.

1d

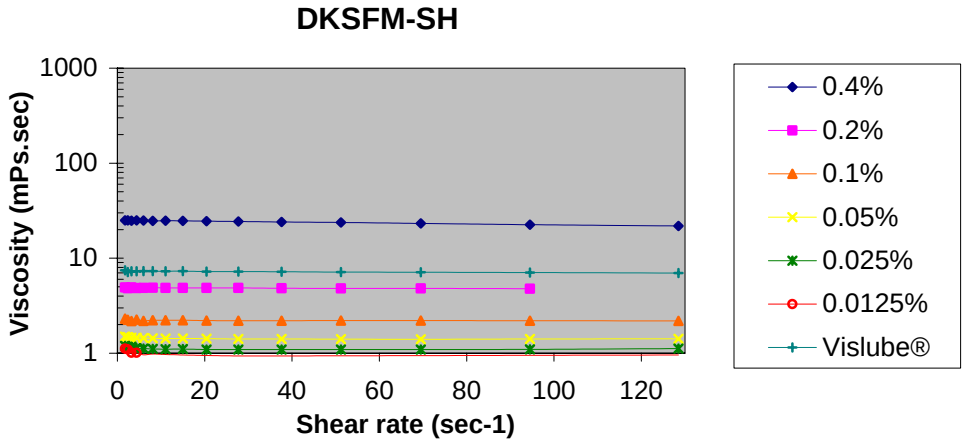


Fig. 1d: DKSFM-SH at all the concentrations and commercial Vislube® have significantly higher viscosity than DKSFM. The viscosity of DKSFM-SH shows concentration-dependent.

1e

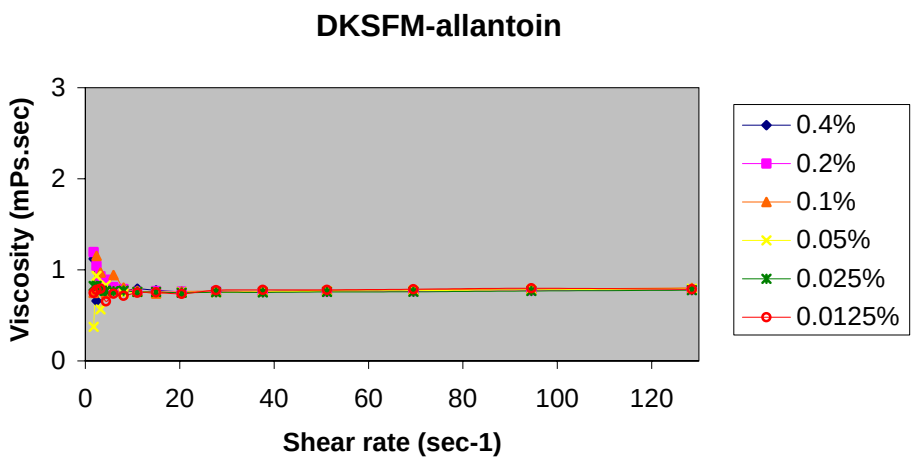


Fig. 1e: The viscosities of DKSFM-allantoin at all the concentrations are close to DKSFM.

1f

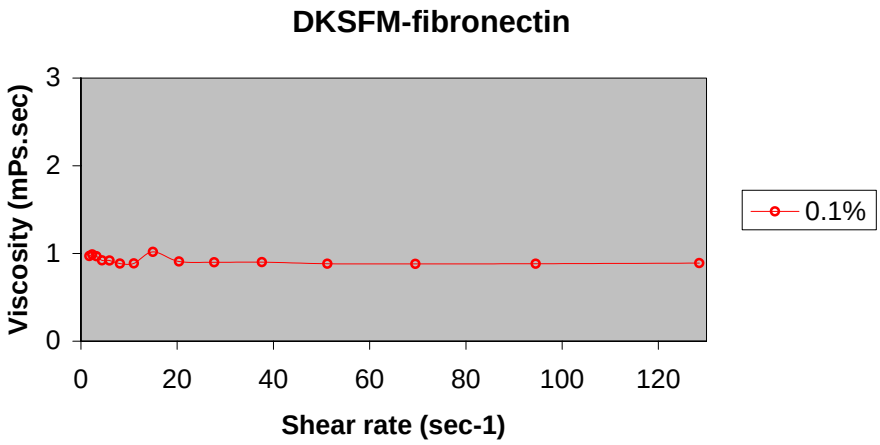


Fig. 1f: Viscosities of 0.1% DKSFM–fibronectin are significantly higher than DKSFM.

3.1.5 Stability of DKSFM-variations after 3 months of storage

In order to investigate their stability, 0.4% solutions of DKSFM-HPMC, -Carbopol, -SH and -allantoin were stored at 4°C for 3 months and the pH, osmolarity, ST and viscosity were remeasured. All the formulations were clear and no sediment was observed after the storage, except for DKSFM-SH at 0.1% and 0.2%, which had turned yellow and obscure because of the obvious bacterial contamination. 0.4% DKSFM-SH was not contaminated and therefore retested after three months. There was no apparent change of pH, osmolarity and ST for all the solutions (Tab. 2).

DKSFM-variations		pH	Osmolarity (mOsm/L)	ST (mN/m)
HPMC	fresh	7.2	290	53.5
	stored	7.2	294	52.6
Carbopol	fresh	7.2	289	80.1
	stored	7.2	303	74.4
SH	fresh	7.2	297	68.7
	stored	7.2	285	69.6
Allantoin	fresh	7.3	308	70.1
	stored	7.3	301	70.6

Tab. 2: Comparison of chemical and physical properties of 0.4% DKSFM-variations before and after 3 months of storage

However, the viscosity of the solutions was found to be altered by long term storage (Fig. 2). The viscosity of 0.4% DKSFM-Carbopol increased after 3 months (P = 0.001) and 0.4% DKSFM-SH decreased (P = 0.001), while the viscosity of 0.4% DKSFM-HPMC was statistically stable (P = 0.069).

2a

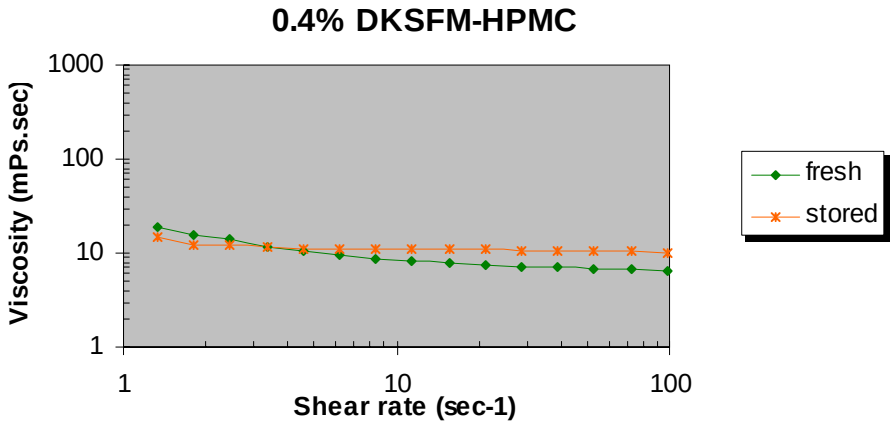


Fig. 2a: Although after the storage the viscosity of 0.4% DKSFM-HPMC decreased slightly after three months below a shear-rate of 4.39 s^{-1} and increased at higher shear-rates, this was not statistically significant ($P = 0.069$).

2b

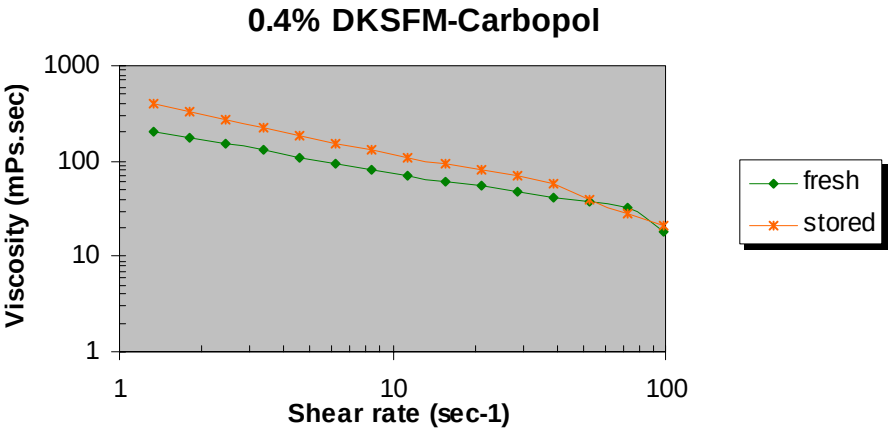


Fig. 2b: The viscosity of 0.4% DKSFM-Carbopol increased after 3 month significantly, $P < 0.05$.

2c

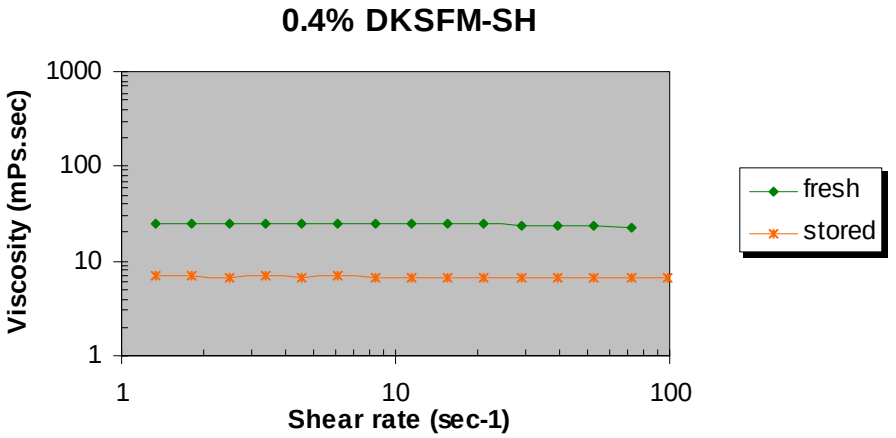


Fig. 2c: The viscosity of 0.4% DKSFM–SH decreased after 3 month significantly, $P < 0.05$.

3.2 NUTRIENT PROPERTIES OF DKSFM-VARIATIONS

3.2.1 Cell culture of primary rabbit corneal epithelial cells

Over the initial 24 h, 70% cells attached to the bottom of the culture flask. After 3 days, the cells were 50% confluent and reached confluent monolayer at day 7. The appearance of primary RCE cells is shown in Fig. 3A, with the typical cobblestone morphology under an inverted phase contrast light microscope.

After passage, the population doubling time was 72 h. The third generation was used in the ATP assay. The forth generation of RCE cells were seeded on Thermanox coverslips and the expression of epithelial cell-specific markers, CK3 and CK19, was investigated using indirect immunohistochemistry staining. Human corneal and conjunctival tissue sections were stained as controls. The full thickness section of a human cornea used as the positive control was CK3+ and the negative control without incubation of primary antibody was CK3-, which indicated no staining background (Fig. 3B). The conjunctival epithelium positive control was CK19+ and the negative control was CK19- (Fig. 3C). The cultured cells stained with CK3 and were negative for CK19 and thus were confirmed to be of corneal origin and character (Fig. 3D).

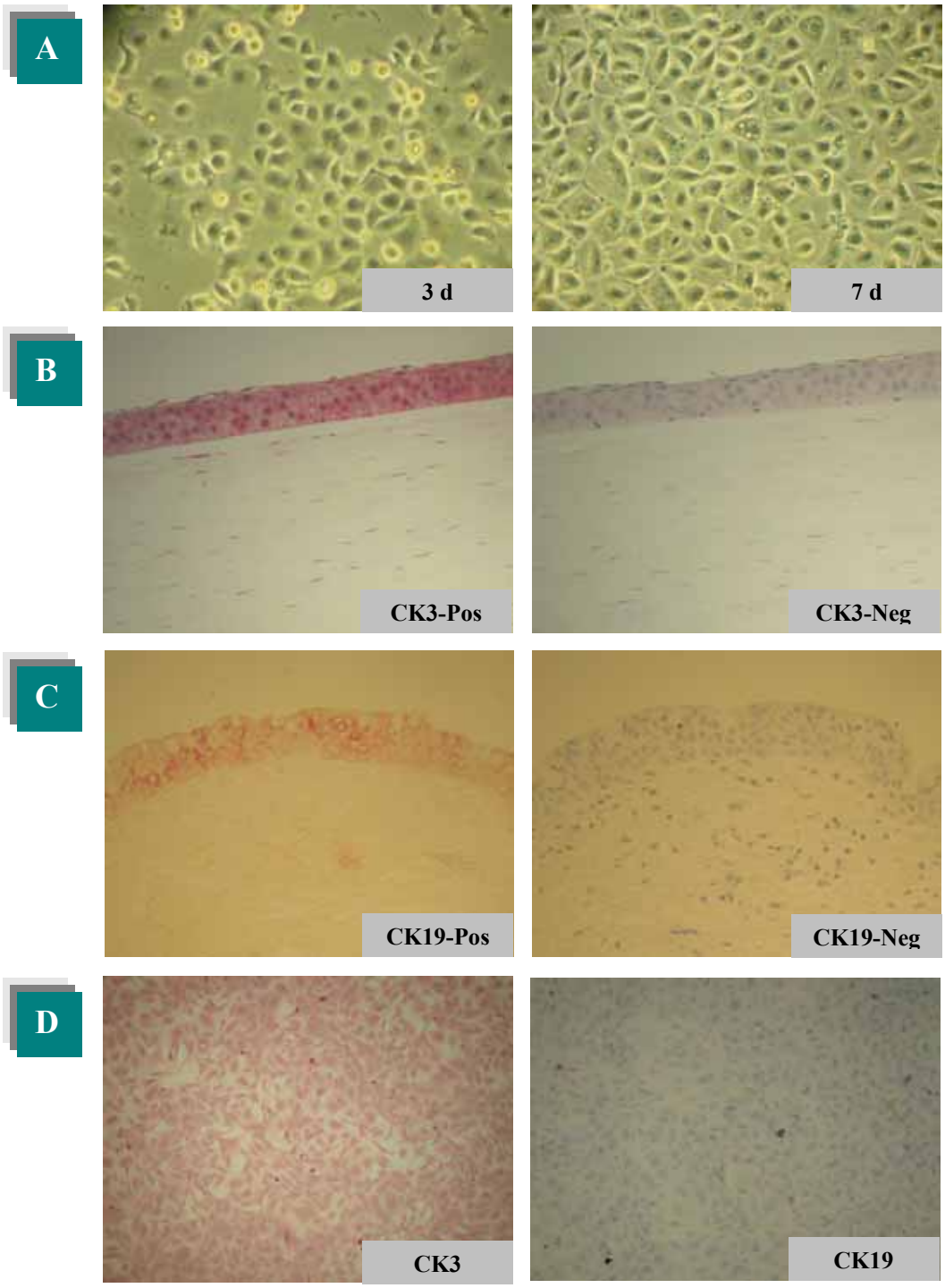


Fig. 3: A) Primary RCE cells at 3 d and 7d;
B) Positive and negative controls of corneal tissue section stained with CK3;
C) Positive and negative controls of conjunctival tissue section stained with CK19;
D) Immunohistochemistry of RCE cells stained with CK3 and CK19.

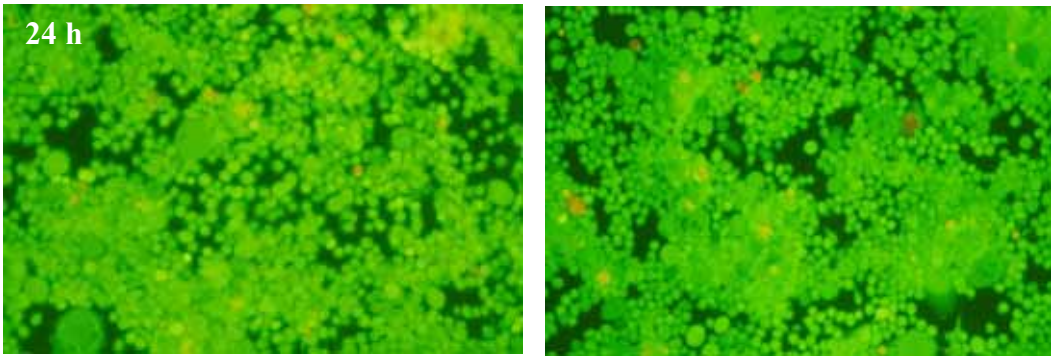
3.2.2 Viability/Toxicity: Calcein AM/EthD-1 assay

Cellular viability was measured with the Calcein AM/EthD-1 assay using the two fluorescent probes, calcein-AM and EthD-1. Corneal (HCE-T) and conjunctival cell line (IOBA-NHC) cells were exposed to test solutions of DKSFM-variations, original and DKSFM diluted commercial artificial tear substitutes for 24 and 72 h. Tab. 5 in the appendix shows percentages of cell viability for both cell lines with the calculations based on fluorescence intensity. The coefficient of variation of the positive control incubated pure DKSFM is 4.8% for HCE-T and 3.5% respectively for IOBA-NHC cells. There is no significant difference in the percentage of cell viability between HCE-T and IOBA-NHC cells for all the solutions tested (P = 0.538). Since fluorescent microscopy also showed similar staining patterns, only the images for IOBA-NHC cells are given in Fig. 4. Green cytoplasmic staining indicates ongoing esterase activity as labelled by calcein-AM and therefore implies that these cells were still viable to some degree. Cells with red nuclear and cytoplasmic fluorescence indicate a damaged cell membrane and were interpreted as dead or nonviable according to the definitions provided by this assay. A third group of cells was present that stained with both labels. However, red nuclear fluorescence shows that the cells were not able to exclude EthD-1, indicating a permeable cell membrane. It should be pointed out that in some wells there were only a few cells left after the staining, because dead cells had become detached from the plastic well bottom during washing. Therefore, the decrease of cells number is an additional indication of toxicity.

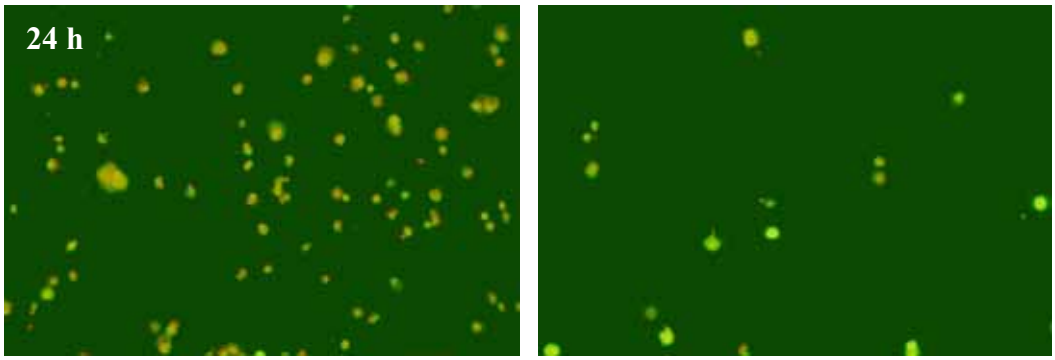
There are no obvious differences between the time points of 24 h and 72 h. All concentrations of 0.1% and lower showed no effect on cell viability compared with the positive control (DKSFM). Most cells were stained green, with a few red cells indicating the normal cell culture heterogeneity. Therefore, only 0.2% and higher concentrations are shown in Fig.4. The peak toxicity was observed at the highest concentration of each substance. DKSFM-HPMC and -allantoin did not modify cellular viability at any concentration at the two time points. However, the commercial Hypromellose® and also 0.2% DKSFM-Hypromellose® damaged cell viability after 24 h. DKSFM-Carbopol showed toxicity after 24 h only at concentrations of 0.2% and 0.4 %. Thilo-Tears® showed higher toxicity than DKSFM-Carbopol. Virtually no decrease in green fluorescence occurred as a result of incubation with 0.2% DKSFM-SH or Vislube®. However, cytotoxicity was found with 0.4% DKSFM-SH after 24h.

Fig. 4a

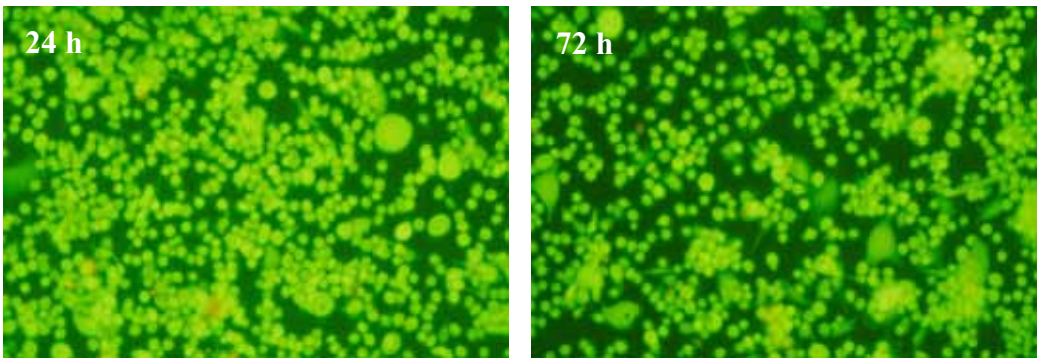
0.2% DKSFM-HPMC



0.2% DKSFM-Hypromellose®



0.4% DKSFM-HPMC



Hypromellose® (0.3% HPMC)

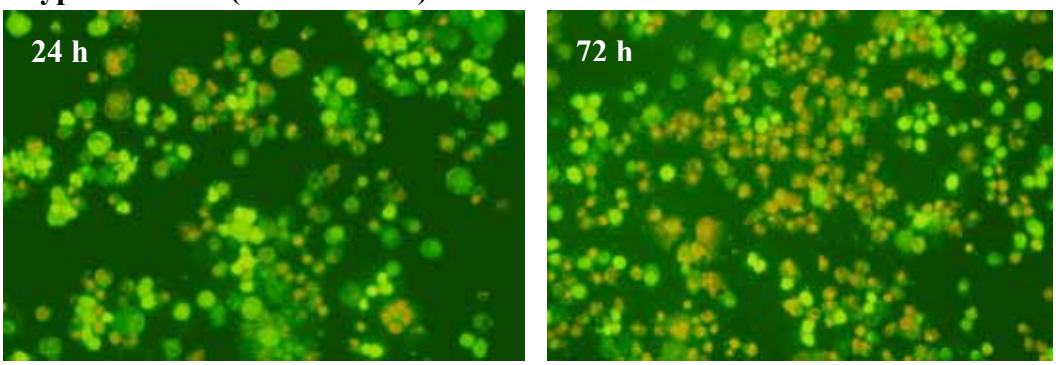


Fig. 4a: The viability of IOBA-NHC cells after incubation with DKSFM-HPMC and Hypromellose® for 24h and 72h. Magnification: ×100

Fig. 4b

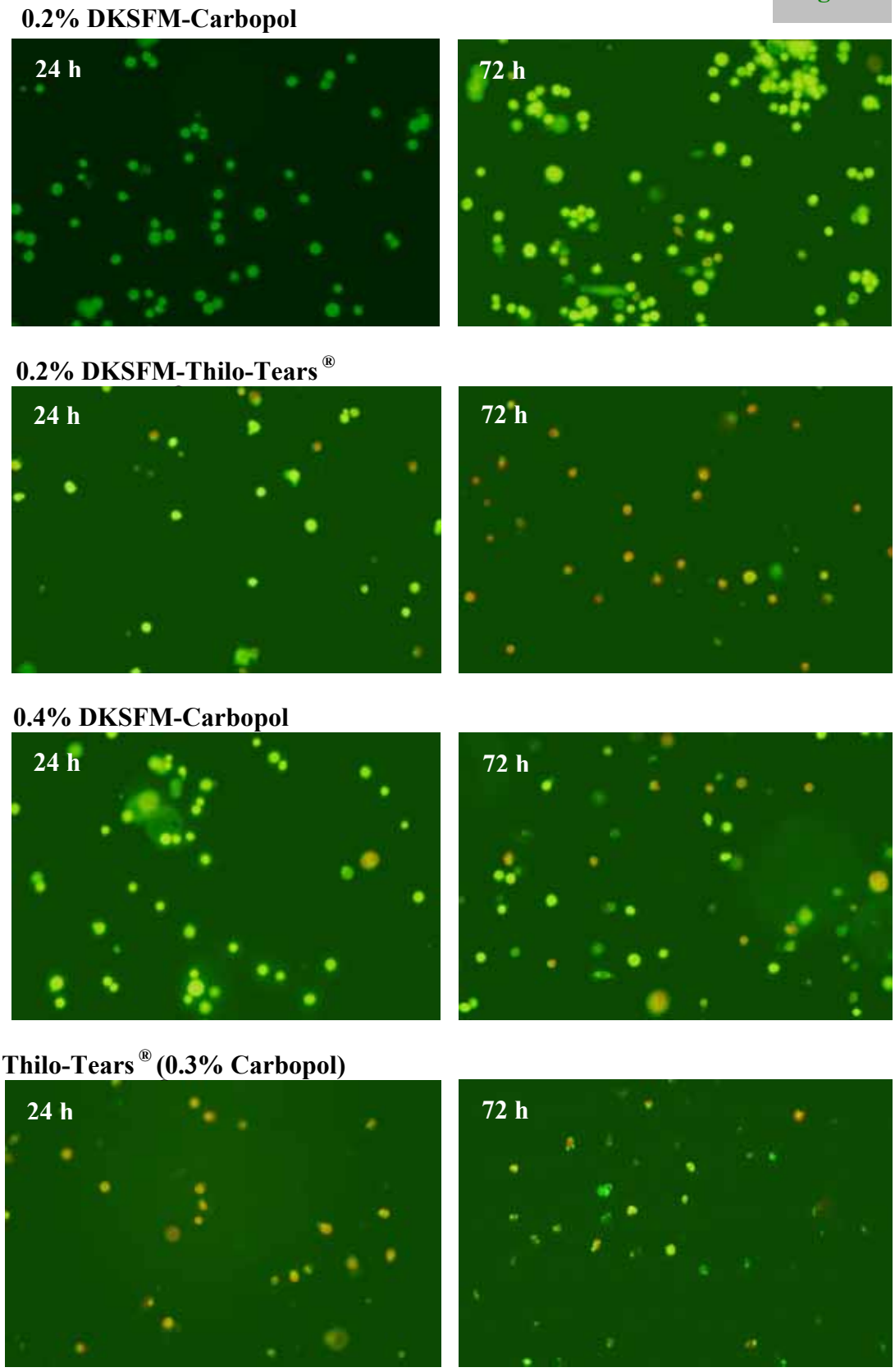


Fig. 4b: The viability of IOBA-NHC cells after incubation with DKSFM-Carbopol and Thilo-Tears® for 24h and 72h. Magnification: ×100

Fig. 4c

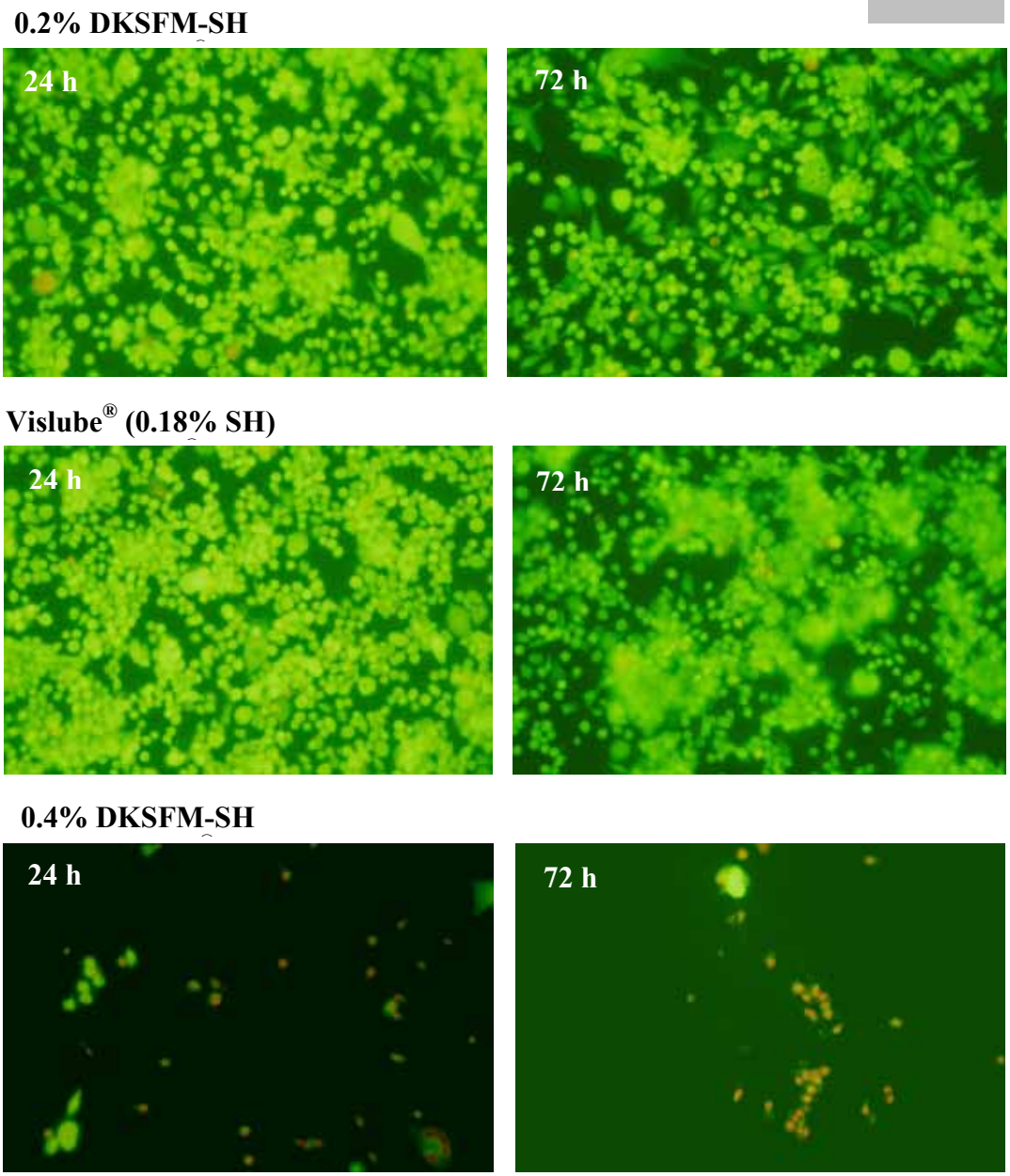


Fig. 4c: The viability of IOBA-NHC cells after incubation with DKSFM-SH and Vislube® for 24h and 72h. Magnification: ×100

Fig. 4d

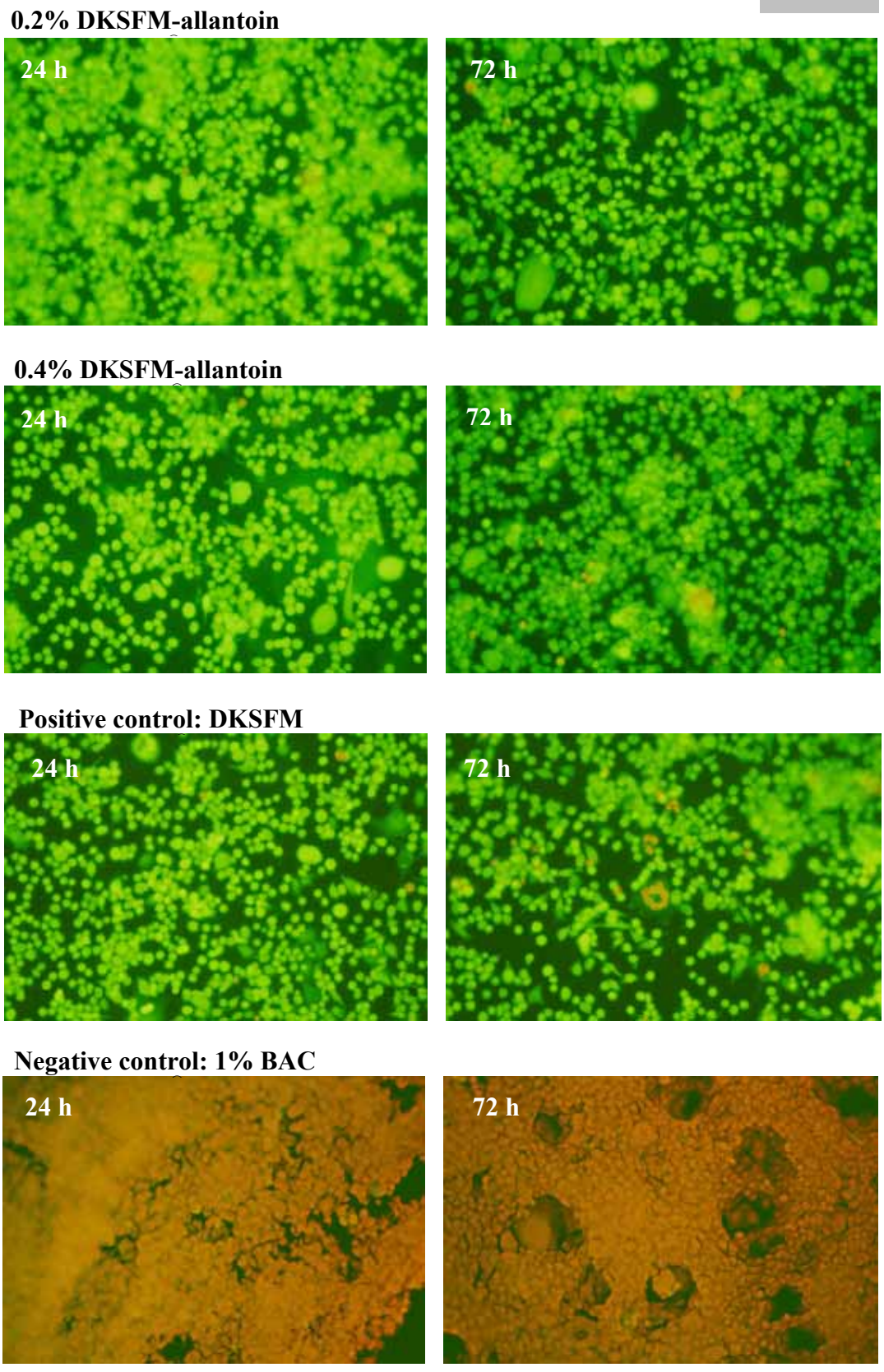


Fig. 4d: The viability of IOBA-NHC cells after incubation with DKSFM-allantoin and controls for 24h and 72h. Magnification: ×100

3.2.3 Proliferation: ATP assay

Dose-response cell proliferation

Fig. 5 and Fig. 6 show the dose-response curves of the ATP assay at the 24 h time point, with the concentrations of DKSFM-variations on the x-axis and relative cell growth on the y-axis. The mean coefficient of variation for positive control cultures with DKSFM was 11.44% which is equivalent to the genetic and phenotypic heterogeneity of normal tissue (Geerling, Daniels et al. 2001) (Cree and Andreotti 1997).

Cell proliferation was best supported by DKSFM-HPMC followed by DKSFM-allantoin, -Carbopol and -SH and these differences were all significant ($P < 0.0001$). The only obvious difference in the response of cellular ATP to test substances was noticed for Chang cells incubated with 0.1% DKSFM-SH. While the ATP content of other cell lines and RCE cells was nearly zero at this concentration, the relative ATP level of Chang cells was 83%. However, such an obvious difference was noted only for this DKSFM-variation and this specific concentration. In general, RCE and CEPI-17-CL4 cells, which are both of corneal origin, were found to be more susceptible to toxicity than HCE-T, Chang and IOBA-NHC cells ($P < 0.0001$). RCE and CEPI-17-CL4 were subjectively found to grow more slowly than the other cell lines and contained less ATP even if incubated with the lowest concentration of DKSFM-variations. This is also confirmed when looking at the area under the curve (AUC) for the dose-response assay on cell proliferation (Tab. 3).

Cell proliferation decreased with increasing concentrations of DKSFM-Carbopol and -SH after 24 h of incubation. While DKSFM-Carbopol began to reduce cell growth at concentrations of 0.2% and resulted in an 80% reduction at the maximum concentration of 0.4%, DKSFM-SH extinguished all ATP at the concentration of 0.1% and higher, except for Chang conjunctival epithelial cell cultures.

Cells incubated with DKSFM-HPMC, -allantoin, or –fibronectin, however, did not show any dose-dependent reduction of ATP. For DKSFM-HPMC at all the concentrations, relative cell growth was around 100%. The relative cell growth incubated with DKSFM-

fibronectin ranged from 74% at 0.005% to 92% at 0.0001% and there was no difference between the different concentrations ($P = 0.09$; Fig. 6).

Fig. 5a

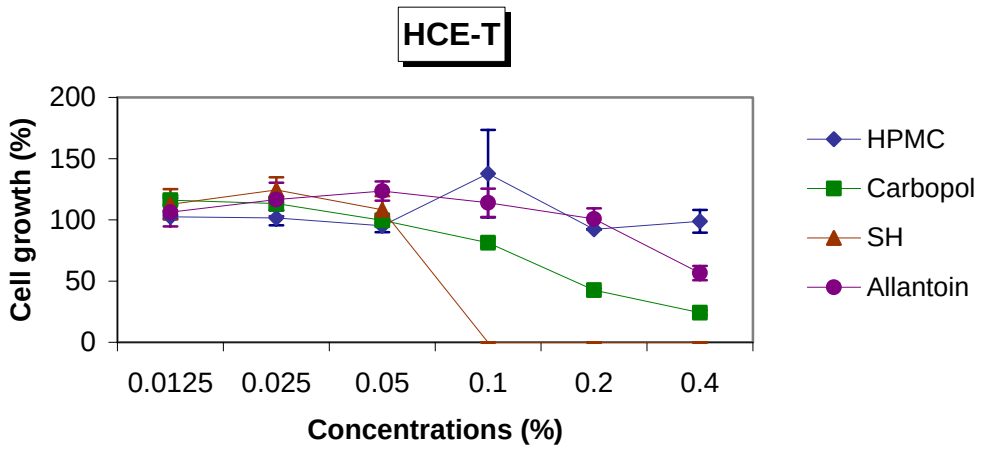


Fig. 5b

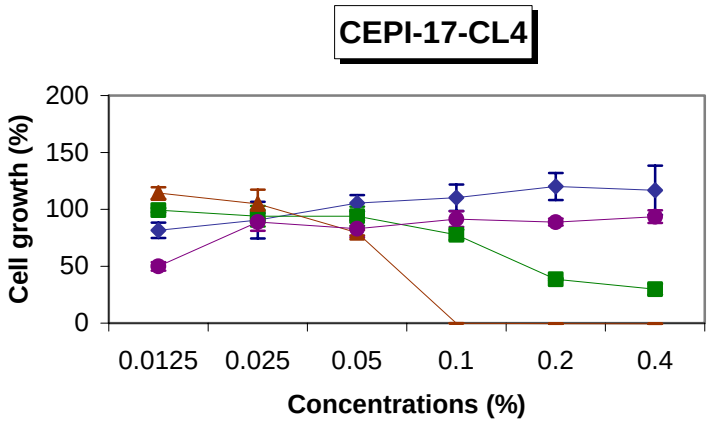


Fig. 5c

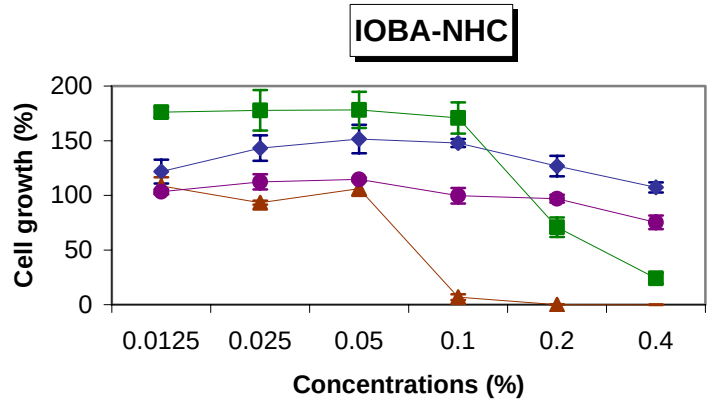


Fig. 5: Dose-response curves of ATP assay of epithelial cells incubated with DKSFM-variations for 24 hours. The error bars are given as Coefficient of Variation (n=3).

Fig. 5d

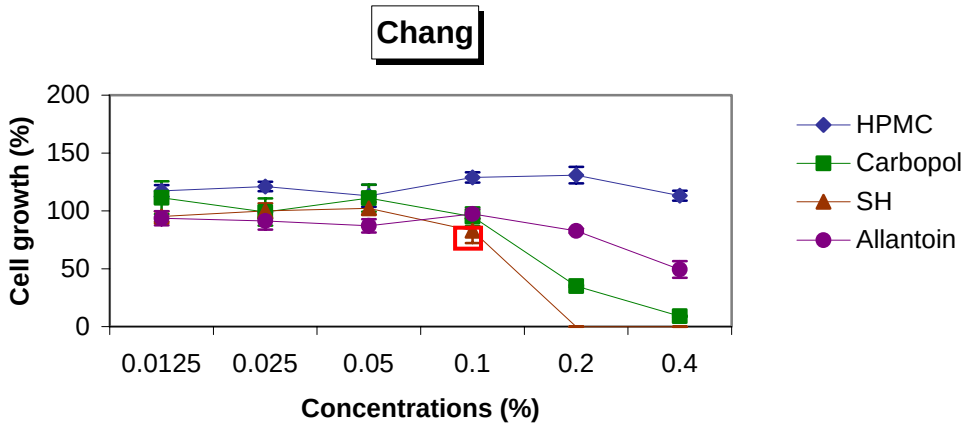
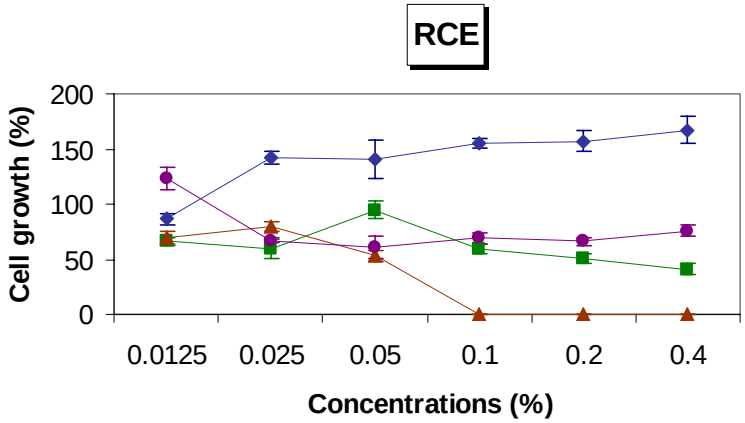


Fig. 5e



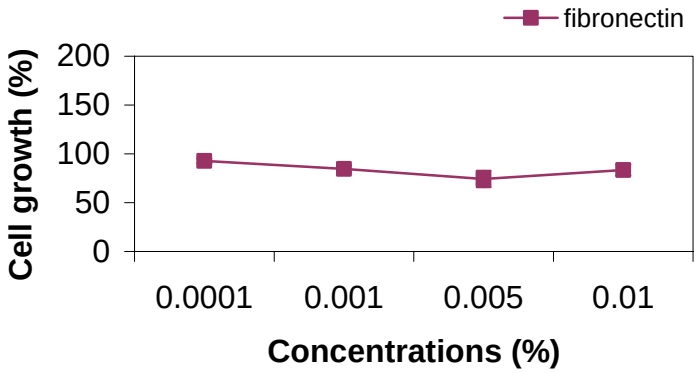


Fig. 6: Cell proliferation of HCE-T cells incubated with DKSFM-fibronectin for 24 hours. The error bars are given as Coefficient of Variation (n=3).

Tab. 3 AUC for the dose-response ATP assay after 24 h incubation

Test substances	Test cells				
	HCE-T	CEPI	Chang	IOBA-NHC	RCE
DKSFM-HPMC	20069	18380	23176	26808	25124
DKSFM-Carbopol	20036	17438	19293	32761	13033
DKSFM-SH	19025	16547	17638	16543	11386
DKSFM-allantoin	21953	15112	17554	20909	15590

Comparison of DKSFM variations and commercial artificial tears

To exclude that any detrimental effects of DKSFM-variations were due to the use of self-prepared laboratory dilutions of HPMC, Carbopol or SH, the cell growth supporting ability of DKSFM-variations was compared with DKSFM supplemented with commercial artificial tears containing the same active component at the equivalent concentration. The importance of these comparative experiments is illustrated by the fact that I observed a significant reduction of ATP levels after incubation with DKSFM-SH, which is not consistent with other published data (Inoue and Katakami 1993). Since in the previous dose-response experiments the toxicity ranking for all solutions tested was identical between the four cell lines and the primary rabbit corneal epithelial cells I performed this second set of ATP-assay experiments with IOBA-NHC cells only which were incubated with all the self prepared and pharmaceutical DKSFM-variations for 24 h.

When compare the mean of the cell growth curve, DKSFM-HPMC supported cell proliferation better than DKSFM-Hypromellose® ($P = 0.028$) and DKSFM-Healon® better than DKSFM-SH ($P = 0.043$), but there was no significant difference between DKSFM-Carbopol and DKSFM-Thilo-Tears® and no difference between DKSFM-SH and DKSFM-Vislube®. The cell growth of the DKSFM-variations at each concentration was also compared and the significant difference was marked with red asterisk (Fig. 7).

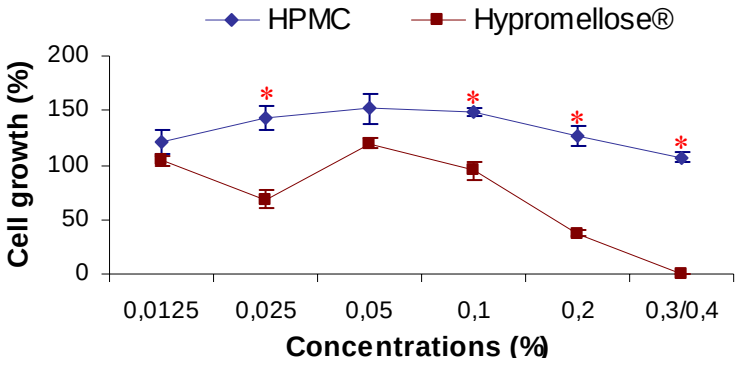


Fig. 7a: Dose-response cell growth curves of IOBA-NHC cells incubated with DKSFM supplemented with HPMC or Hypromellose® for 24 hours. The error bars are given as coefficient of variation (n=3). From this the beneficial effect of using DKSFM as diluent becomes obvious.

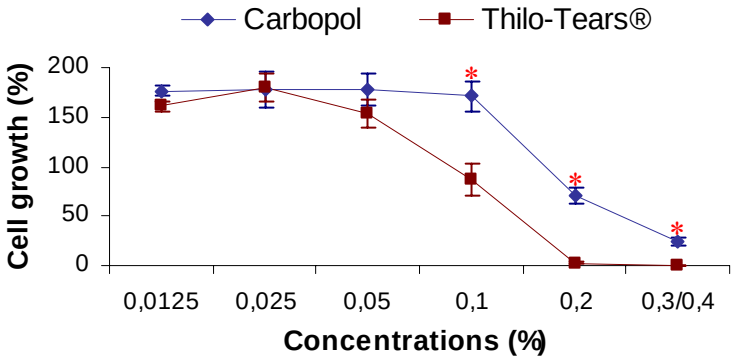


Fig. 7b: Dose-response cell growth curves of IOBA-NHC cells incubated with DKSFM-Carbopol, DKSFM-ThiloTears® or undiluted ThiloTears® (0.3% Carbopol) for 24 hours. The error bars are given as Coefficient of Variation (n=3).

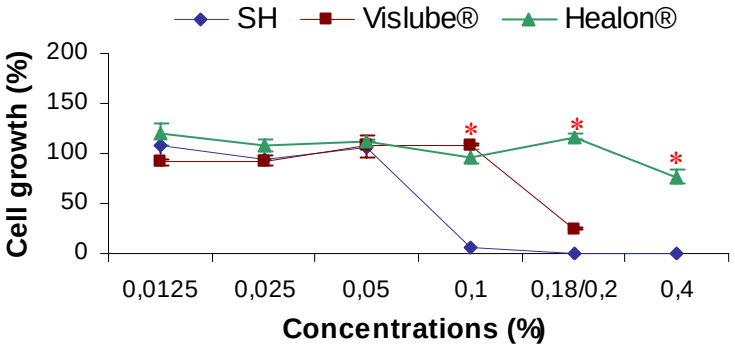


Fig. 7c: Dose-response cell growth curves of IOBA-NHC cells incubated with DKSFM-SH, undiluted Vislube® (0.18% SH) and Vislube® or Healon® diluted in DKSFM for 24 hours. The error bars are given as Coefficient of Variation (n=3).

Time-response cell proliferation

Time-response ATP assays were performed for the four cell lines for 24, 48, 72, 96 and 144 h with all DKSFM-variations. The results are presented in appendix Tab. 4. Relative cell growth curves of HCE-T (corneal epithelial) and Chang (conjunctival epithelial) cells treated with 0.2% DKSFM-variations are given in Fig. 8 as representative examples.

In general, cell growth showed a time-dependent manner with DKSFM-variations. The peak toxic effect with all the formulations was often seen at the top concentration after the longest incubation time. Significant differences of cell proliferation among the DKSFM-variations were found ($P < 0.0001$). Cell growth was better supported by DKSFM-HPMC, then DKSFM-allantoin, -Carbopol and -SH subsequently.

CEPI-17-CL4 cells showed more susceptibility than other cell lines when treated with DKSFM-HPMC. DKSFM-HPMC had little effect on cell growth after 24, 48 and 72 h. The decrease of cell proliferation was observed with CEPI-17-CL4 cells after incubation with DKSFM-HPMC for 96 h, but other cell lines for 144 h. The time-dependent manner was obviously observed with 0.2% and 0.4% DKSFM-Carbopol, which increased cell damage from 70% after 24 h to 100% after 144 h. DKSFM-Carbopol of lower concentrations could still support more than 50% cell growth over 144 h. DKSFM-SH induced cell death with concentrations higher than 0.1% at any exposure time. It was surprisingly noticed that cell growth HCE-T cells were better supported by 0.1% DKSFM-SH after 96 h (72%) than 72 h (0%). No obvious alteration of cellular viability was observed with DKSFM-allantoin, except with concentrations higher than 0.1% for longer than 72 h treatment.

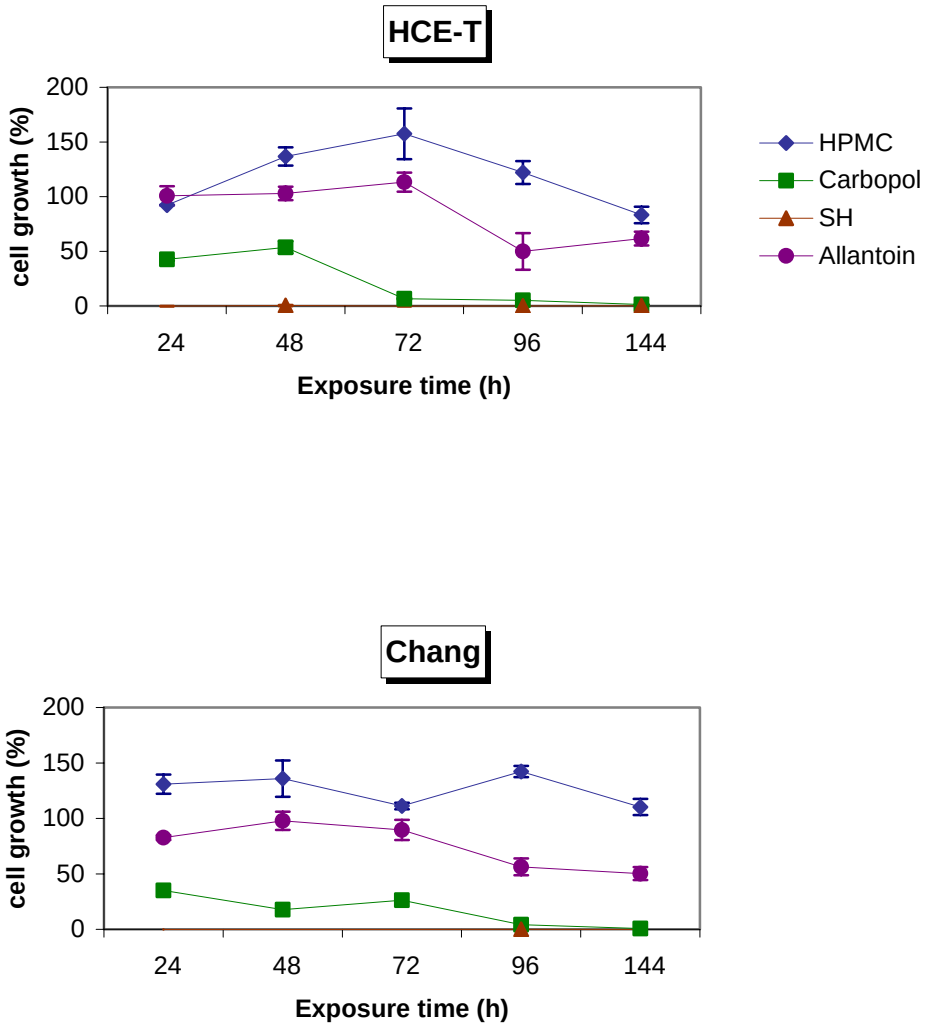


Fig. 8: Time-response curves of ATP assay of HCE-T and Chang cells incubated with 0.2% DKSFM-variations. The error bars are given as Coefficient of Variation (n=3).

Cell growth support of DKSFM-variations after 3 months of storage

DKSFM-HPMC, -Carbopol, -SH and -allantoin with the top concentration, 0.4%, were stored at 4°C for 3 months. Besides the measurements of pH, osmolarity, ST and viscosity, proliferation of HCE-T cells was retested with 0.4% DKSFM-variations for 24 h exposure and compared with the fresh formulations (Fig. 9). All cells died after incubation with both fresh and stored 0.4% DKSFM-SH for 24 h, but the other DKSFM-variations were able to maintain cell viability. There was no significant difference between the ATP levels of HCE-T cells incubated with fresh and stored DKSFM-HPMC, -Carbopol, or -allantoin.

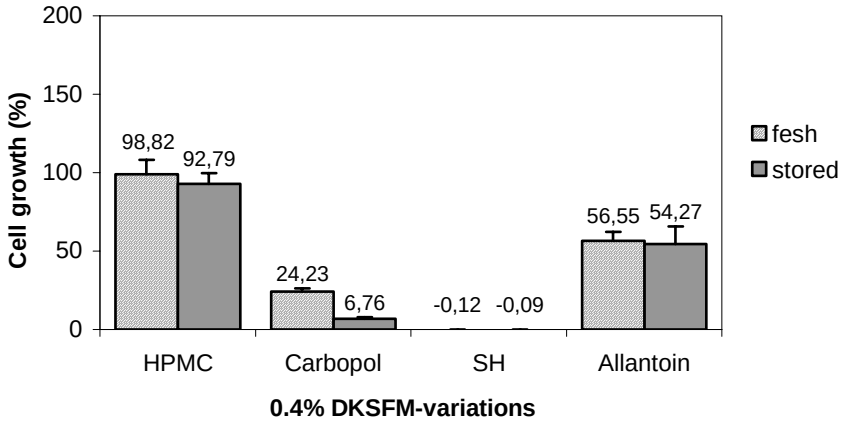


Fig. 9: Comparison of proliferation of HCE-T cells incubated with fresh and 3 months' stored 0.4% DKSFM-variations for 24 hours. The error bars represent the coefficient of variation (n=3).

3.2.4 Relevance of cell viability test systems

Both ATP assay and Calcein AM/EthD-1 assay were applied to examine the effect of DKSFM-variations and artificial tears on IOBA-NHC cells after 24 hours of incubation. The results are presented in Tab. 6 of the Appendix. The concentrations at which the cell viability was obviously reduced are highlighted in red. The two assays showed a similar trend in detecting toxicity. The ATP assay detected cytotoxicity of DKSFM-Carbopol, -SH, -Thilo-Tears® and -Vislube® at lower concentrations than the Calcein AM/EthD-1 assay. The numbers of samples at concentrations ≤ 0.2% detected toxic by the two assays are shown in Tab.4. The ATP-assay detected toxicity for significantly more test-solutions than the Calcein AM/EthD-1 assay ($P = 0.007$) as analysed with the Chi-square test.

	ATP assay	Calcein AM/EthD-1
Detected	5	2
Not detected	1	4

Tab. 4: Number of samples in which toxicity was detected by ATP assay or Calcein AM/EthD-1 assay after 24 hours of incubation at ≤ 0.2% of test substances.

3.2.5 Migration: Colony dispersion assay

A corneal epithelial cell line, CEPI-17-CL4, and a conjunctival epithelial cell line, Chang, were used in a colony dispersion assay to assess migration. Two concentrations of each DKSFM-supplement were tested in this assay based on the results of the viscosity measurement, the ATP assay and the Calcein AM/EthD-1 assay. In general, I chose to test concentrations of the DKSFM-variations with the highest viscosity that was not associated with a substantial toxicity. Therefor, 0.2 % and 0.4% DKSFM-HPMC, 0.05% and 0.1% DKSFM-Carbopol as well as 0.05% and 0.1% DKSFM-SH were used with the higher viscosity and lower toxicity. Since allantoin could not adjust the viscosity of DKSFM, less toxic concentrations, 0.025% and 0.05%, were used.

Fig. 10 shows the colony area of cells after incubation with the above DKSFM-variations for 24, 48, 72, 96 and 144 h. The colony areas are the mean of triplicates. The Chang cells

did not migrate with any of the test solutions. The migration of CEPI-17-CL4 cells incubated with 0.2% (P = 0.02) and 0.4% DKSFM-HPMC (P = 0.04), 0.05% DKSFM-Carbopol (P = 0.004) and 0.05% DKSFM-allantoin (P = 0.007) increased over 144 h. A drop of the curve was noticed with 0.1 % DKSFM-SH because of its cytoxicity, also shown in the ATP assay, the dead cells subsequently being lost during washing steps.

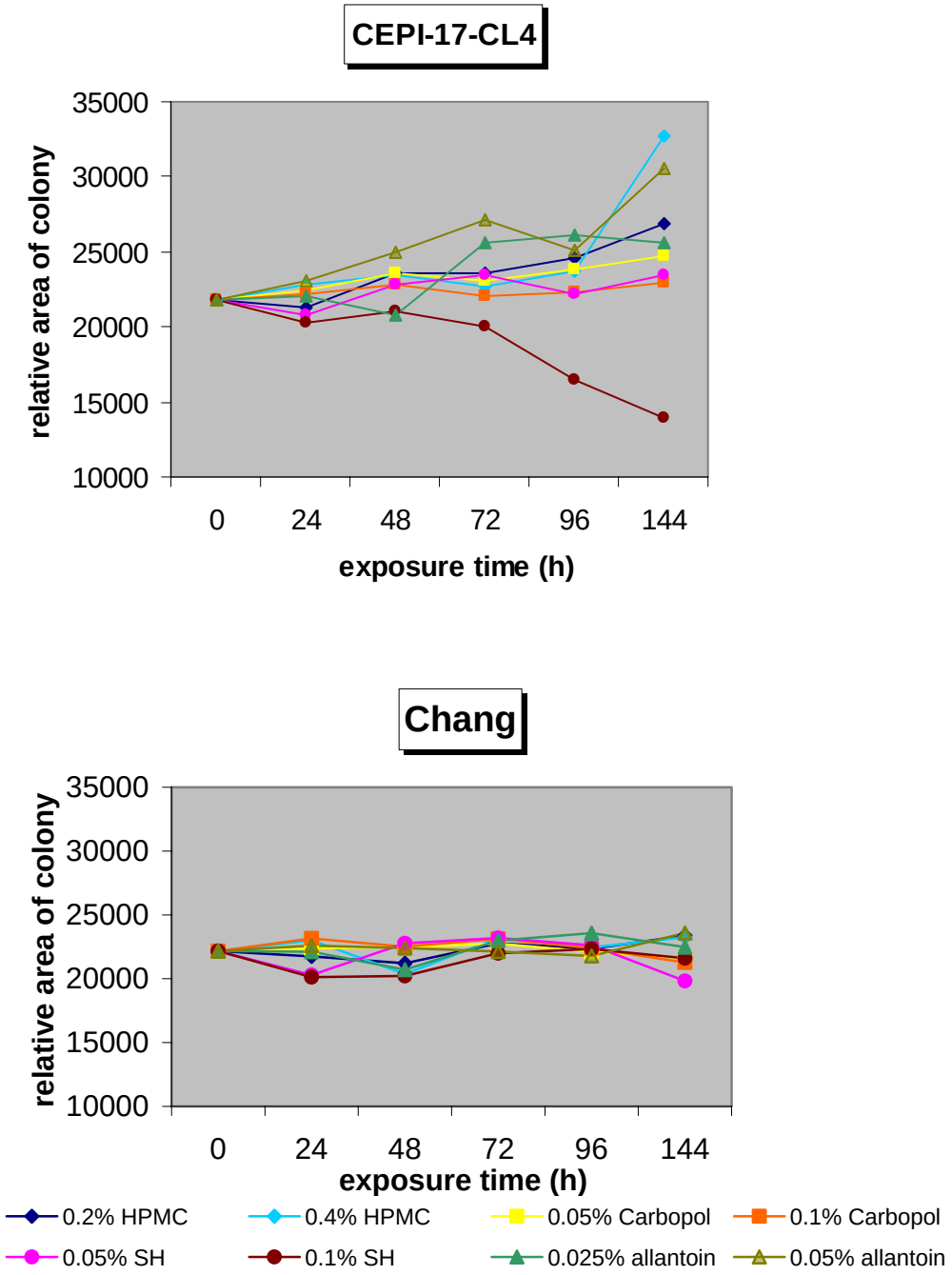


Fig. 10: Colony dispersion assay of CEPI-17-CL4 and Chang cells treated with DKSFM-variations from 24h to 144 h.

A number of previous studies described that SH enhances corneal epithelial growth and migration (Miyazaki, Miyauchi et al. 1996) (Miyauchi, Sugiyama et al. 1990). I thus evaluated the migration-stimulating ability also for Healon® dissolved in DKSFM to the same concentrations as other DKSFM-variations, 0.0125%, 0.025%, 0.05%, 0.1%, 0.2% and 0.4%. CEPI-17-CL4 cells were incubated in DKSFM-Healon® for 72 h and migration was improved by all the concentrations compared to zero h (P = 0.028, Fig. 11) however no dose-response correlation was observed.

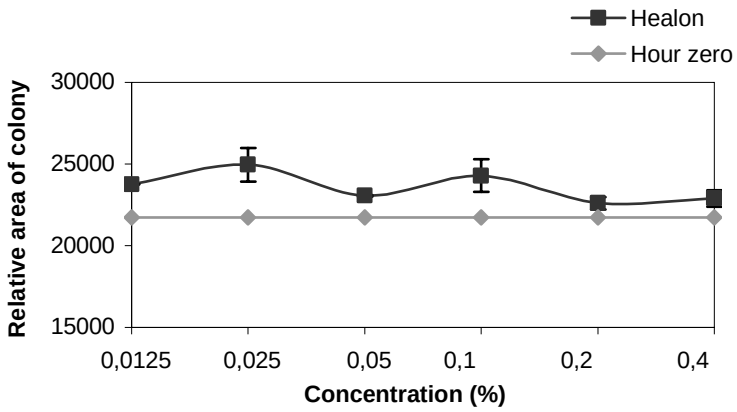


Fig. 11: Colony dispersion assay showed that CEPI-17-CL4 cell migrated when treated with DKSFM-Healon® for 72 h.

The concentrations of 0.0001%, 0.001%, 0.005% and 0.01% DKSFM-fibronectin were also tested with this assay. Compared with hour zero, DKSFM-fibronectin did not improve CEPI-17-CL4 cell migration after 72 h (P = 0.068). (Fig. 12)

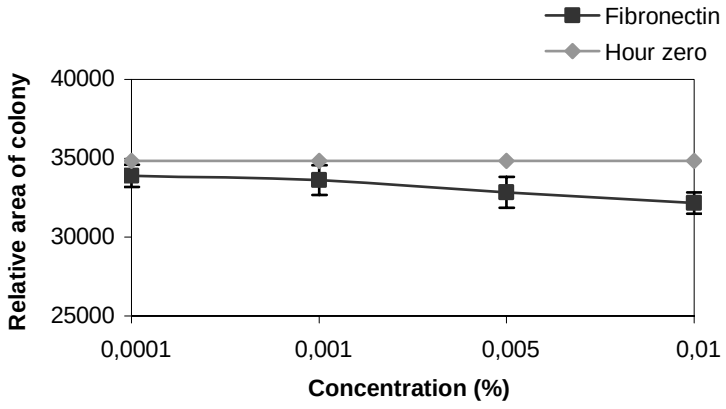


Fig. 12: Colony dispersion assay of CEPI-17-CL4 cells treated with DKSFM-fibronectin for 72 h.

4 DISCUSSION

Tear replacement with topical artificial tear substitutes and lubricants is currently the most widely used therapy for dry eye. Most preparations consist of a solution containing electrolytes, surfactants, viscosity agents and preservatives. This combination of ingredients strives to strike an ideal balance between maximizing tear film stability and ocular surface retention while simultaneously minimizing vision-blurring or caking. HPMC, Carbopol, SH, and polyvinyl alcohol are all commonly seen examples of viscosity agents in current artificial tears. Some artificial tears employ a combination of two or more such agents. A number of nutrients have been used in artificial tears, such as dextrose, sodium lactate, vitamins (Gilbard, Huang et al. 1989), growth factors (Daniele, Gilbard et al. 1992) and hormones (Sullivan, Ariga et al. 1994). However, they can not reproduce the components exactly or substitute the complex composition of natural tears. In this thesis I describe the first attempt to use a defined serum free medium, DKSFM, as a base solution to formulate a nutrient and lubricant tear substitute. DKSFM contains a wide range of hormones, amino acids, vitamins and growth factors and can maintain morphology and physiological properties of primary corneal epithelial cells *in vitro*.

A variety of properties have been regarded as important factors in the formulation of tear substitutes including pH, osmolarity, viscosity and ST. Since DKSFM is designed for culture of keratinocytes, its pH and osmolarity are already optimised to support epithelial cell growth. To improve the wetting properties and retention time on the ocular surface, the surface tension and viscosity of any tear substitute should be optimised. The viscosity of DKSFM (0.71 mPa.sec) is already lower than that of human tears (5 mPa.sec) at a shear rate of 2 sec^{-1}, while the viscosity of commercial artificial tears such as Vislube® (233 mPa.sec) usually is much higher. Therefore, HPMC, Carbopol and SH were tested as supplements to increase the viscosity of DKSFM. Their effect on the ST of the medium was also investigated. The ST of DKSFM was initially higher (71 mN/m) than the data reported for the tear film (43 mN/m for unstimurlated and 46 mN/m for stimulated tears) (Pandit, Nagyova et al. 1999) (Tiffany, Pandit et al. 1998). Fibronectin and allantoin were also tested due to their reported nutrient potential (McCulley, Horowitz et al. 1993) (Nishida, Ohashi et al. 1983) (Spigelman, Deutsch et al. 1987). Of course, tear substitutes should be little or not toxic at all, since they otherwise may either further worsen the ocular

surface disease directly or boost the underlying inflammatory condition (Burstein 1985) (Tripathi and Tripathi 1989; Tripathi, Tripathi et al. 1992). The effect of all the formulations prepared on viability, proliferation and migration of corneal and conjunctival epithelial cells was thus tested.

4.1 BIOCHEMICAL AND BIOPHYSICAL PROPERTIES OF DKSFM-VARIATIONS

The main purpose for measuring the chemical properties was to exclude an unphysiological level of pH or osmolarity, which in themselves can induce toxicity (Geerling, Daniels et al. 2001) (Worth and Cronin 2001).

4.1.1 PH

Normal human tears have a mean pH value of 7.45 +/- 0.16 (Carney and Hill 1976). Most commercial preparations vary in their polymer content and viscosity, but tend to be isotonic with a pH ranging from neutral to acid. Many patients with KCS complain that acid tear substitutes burn and tend to aggravate rather than relieve their ocular discomfort. Using a bicarbonate buffer or borax-boric acid buffer system, alkaline eye substitute with a pH of approximately of 8.4 can be prepared, which are well tolerated by KCS sufferers and give the subjective relief (Jones and Voop 1965) (Wright 1971) (Raber and Breslin 1978) (Motolko and Breslin 1981).

HPMC, SH, allantoin and fibronectin did not change the pH of the basic medium, which remained between 7.2 and 7.3. When Carbopol was first added to DKSFM, the pH dropped from 7.2 to about 3.0 due to the free acrylic acid groups which lead to high activity of hydrogen ion. Increased ionic strength of polyacrylate acid at low pH reduces its viscosity. Therefore, in order to achieve maximum viscosity, the pH of a Carbopol containing solution must be neutralized to 7.0. The viscosity of Carbopol begins to decrease again after a pH of 9.0, and will continue to decrease if the pH is increased further. This is due to the dampening of the electrostatic repulsion caused by the presence of excess electrolytes. I used NaOH as a neutralizer, since the most common way to achieve maximum thickening with Carbopol polymers is by converting the acidic polymer to a salt. DKSFM-Carbopol

was adjusted to the physiological pH of 7.2 and only DKSFM-variations with pH 7.2 or 7.3 were used in this *in vitro* study. However, alkaline eye drops with a pH less than 9.0 could be used in later clinical trials to further improve patients' comfort.

4.1.2 Osmolarity

Osmolarity is the concentration of osmotically active particles expressed in terms of osmoles of solute per litre of solution. Osmole is the molecular weight of a solute, in grams, divided by the number of ions or particles into which it dissociates in solution. When two solutions of different concentrations of a solute are separated by a membrane, there is a net diffusion of the solvent across the membrane towards the side with the higher concentration which is a phenomenon used by the ophthalmologist when he uses hypertonic saline to dehydrate an oedematous cornea. The osmolarity of human serum is 290 mOsm/L, equivalent to a 0.9% NaCl aqueous solution. The osmolarity of normal human tears is about 300 mOsm/L (Gilbard, Farris et al. 1978). However, a wide interindividual and intraindividual variation exists.

In this study, the osmolarity of the basic medium, DKSFM, was 278 mOsm/L. The osmolarities of DKSFM-HPMC, -Carbopol, -SH and -allantoin ranged from 276 to 308 mOsm/L after adjusting the pH to 7.2-7.3. I did not adjust the osmolarity for any of these formulations, since the osmolarity values were close to the normal human tears and acceptable for cell culture. The osmolarity of 0.1% DKSFM-fibronectin was 569 mOsm/L, which would be toxic in cell culture (Geerling, Daniels et al. 2001). This dropped rapidly after dilution to lower concentrations, which indicates the influence of solute concentrations on the osmolarity of the solution. I could not explain why 0.1% (1 mg/mL) DKSFM-fibronectin has such high osmolarity compared to DKSFM, since the molecular weight of fibronectin is about 405 kD and theoretically the osmolarity of 405,000 mg/mL fibronectin is only 1000 mOsm/L.

The osmolarity of a drop applied to the eye is very important for its subjective perception. The osmolarity of tears changes when the normal tear film structure is altered such as in dry eye and contact lens wearers (Bron, Tiffany et al. 2002; Iskeleli, Karakoc et al. 2002). Tear hyper-osmolariy occurs either as a result of evaporation in the presence of reduced tear flow, or excessive evaporation in the presence of a normal flow rate. Tear osmolarities

of individuals with KCS and normal controls were compared by different authors and although the results varied, osmolarity was generally found to be higher in dry eyes, 323-343 mOsm/L for KCS and 302-306 mOsm/L for normal controls (Gilbard, Farris et al. 1978; Lucca, Nunez et al. 1990). In KCS a significant positive correlation between tear film osmolarity and Rose Bengal staining of ocular surface damage has been shown (Gilbard and Farris 1979). Therefore, hypo-osmolar formulations are often used to improve the environmental conditions of the ocular surface and to relieve dry eye symptoms. Aragona et al reported that 150 mOsm/L SH containing eye drops improved dry eye syndromes significantly better than isotonic SH (Aragona, Di Stefano et al. 2002) and Iester et al also reported improvements in BUT, vital staining and Schirmer test of dry eyes after using hypotonic 0.4% hyaluronic acid eye drops (Iester, Orsoni et al. 2000). However, Wright et al found that preparations of BJ6 and hypromellose of 160 mOsm/L were not more effective than their iso-osmolar equivalents (Wright, Cooper et al. 1987). Also, Papa et al showed no difference in dry eye symptoms relief between a hypotonic SH solution of 215 mOsm/L compared with an isotonic SH solution of 305 mOsm/L. Since the benefit of hypo-osmolar artificial tears for KCS is still controversial, an iso-osmolar tear substitute should be used in any initial clinical trial of a chosen DKSFM-variation.

These data show clearly that the pH and osmolarity of all DKSFM-variations tested were in the physiological range and therefor unlikely to induce any toxicity or altered cell behaviour.

4.1.3 Surface tension

ST is defined as the force acting on the surface of a liquid that tends to minimize the area of the surface, or the force that appears to act across a line of unit length on the surface, also known as interfacial force, interfacial tension or surface tensity. The cohesive forces between liquid molecules are responsible for this phenomenon. The coating ability of the substance can be characterized to some extent by measuring the ST. When a liquid spreads on a solid, any liquid with a ST below the critical ST of the solid will result in total wetting, while liquid with a ST above the solid will maintain the feature of a drop. From this it is obvious that ST is an important physical property of tears, since it will influence the formation and stability of the preocular tear film.

The ST of water at 32°C is 71 dynes/cm, of unstimulated human tears 43 mN/m, and of stimulated tears 46 mN/m (Pandit, Nagyova et al. 1999) (Tiffany, Pandit et al. 1998). DKSFM-Carbopol, -SH, -allantoin, and -fibronectin as well as Thilo-Tears® and Vislube® were close to that of water. However, ST values of DKSFM-HPMC at all the concentrations and commercial Hypromellose® eye drops were around 50 mN/m. It seems striking, that no effect of the concentration on surface tension was detected for any of the solutions tested. Since the electronic manometer used in this study measures the ST only for the air-liquid interface, the molecules of accumulate at this air-liquid interface in higher concentration than in the solution itself, thus resulting in a relatively stable ST measurement. Of all substitutes tested only HPMC was able to reduce the ST of DKSFM. It is unclear why this happens, but theoretically the HPMC-molecules could disrupt or modify the hydrogen bonding network of the water-based DKSFM.

Holly et al. reported that the critical ST of the corneal epithelium was 28 mN/m (Holly and Lemp 1971), but the methods used were found to cause denaturation of the corneal epithelium (Cope, Dilly et al. 1986). Later Tiffany reported that the surface tension of intact cells is much closer to that of water, the cornea/tear system is therefore close to a total wetting situation (Tiffany 1990). ST of HPMC containing solutions is close to human tears and much lower than the critical ST of the corneal epithelium, indicating that HPMC would offer a superior coating ability and minimal disruption of the tear film.

The retention time is influenced by viscosity, bioadhesion and ST. Although a lower ST indicates a better ability to coat, at present, not all of the factors involved in coatability are known. Bioadhesion, and more specifically mucoadhesion, is an important behaviour of polymers to attach to the glycoproteins on epithelial surfaces. Mucoadhesion is enhanced by polyanionic polymers, such as HPMC, Carbopol and SH (Smart, Kellaway et al. 1984) (Mortazavi and Smart 1994). Theoretically, the more negatively charged substance has a greater affinity for positively charged tissues, instruments and implants (Harrison, Soll et al. 1982). SH has several desirable attributes such as pseudoplastic rheology and good bioadhesion, but it is costly to produce in commercial quantities. Compared to SH, HPMC has also excellent mucoadhesion and is inexpensive and readily available.

4.1.4 Viscosity

Viscosity is a measure of a solution's resistance to flow and is a function of molecular attraction that resists flow. It is defined in units called Pascal·second (Pa·s), which is identical to 1 $N \cdot s/m^2$ or 1 kg/m·s, and is the ratio of the shearing stress to the velocity gradient in a fluid. Increased viscosity can increase retention time, reduce the drainage rate and increase the bioavailability on the ocular surface. A long contact time, achieved by a high viscosity of the tear substitute is usually favourable, since this reduces the number of applications required to control symptoms and signs. HPMC, Carbopol and SH are used in many artificial tear formulations to increase viscosity and their topical application has been shown to confer both subjective and objective relief in patients with dry eye syndromes (Debbasch, Pisella et al. 2000; Aragona, Papa et al. 2002).

I attempted to adjust the viscosity of DKSFM by supplementing it with the major physically active compounds of the most common type of equivalent commercial products, which are HPMC, Carbopol, and SH. The measurements showed that these three substances increased the viscosity of DKSFM as expected even at the low concentration of 0.0125%. The viscosity of 0.4% Carbopol was extremely high, with 205 mPa.sec at at 2 sec^{-1} and 18 mPa.sec at 128 sec^{-1} shear rate. A solution with such a high viscosity will not mix with natural tears and will not be well tolerated. Carbopol is therefore sometimes combined with polyvinylpyrrolidone (PVP) as a 2^{nd} polymer to reduce the viscosity but achieve a high mucoadhesion at the same time (Oechsner and Keipert 1999).

The viscosity of the DKSFM with the single compound was comparable to the equivalent commercial artificial tear substitutes tested. Ideally, tear substitutes have a high enough viscosity capable to prolong the contact time, but low enough not to produce dragging sensations and epithelial damage with rapid eye or lid movements (Doane 1980). Highly viscous tear substitutes with viscosity above 200 mPa.sec are inadequate for lubrication because they make blinking difficult and form lumps that disturb vision. Although the optimal range of viscosity for clinical use has not been evaluated systematically, mild to moderate viscous tear agents are best for this purpose.

The viscosity of a polymer solution is a composite function of the molecular weight and the concentration, as well as the temperature and solvent used (Bothner and Wik 1987). Since the temperature during the measurements was set to 37°C and controlled by a water circulating device and one manufacturer and molecular weight were used for each supplement throughout the study, the viscosity of DKSFM-variations depended on the concentration only and increased with higher concentrations. DKSFM-HPMC with concentrations from 0.2% to 0.4% and DKSFM-SH at concentration of 0.4% demonstrated moderate viscosity and might be suitable for use in a clinical trial, which needs to be performed to assess the retention time and clinical efficacy of these formulations *in vivo*.

A visco-elastic fluid can be non-Newtonian or Newtonian character, depending upon whether or not the viscosity changes with the shear rate. The viscosity of a non-Newtonian fluid is high under conditions of low shear rate and low under conditions of high shear rate, also described by the term “shear-thinning” or “pseudoplasticity”. Water and silicone oils are Newtonian fluids and have a constant viscosity regardless of the shear rate, but normal human tears are a non-Newtonian fluid, with viscosity falling from about 5 mPa.sec at 2 s^{-1} to about 1.5 mPa.sec at 160 s^{-1} (Tiffany 1991). Tiffany’s present work indicates that the viscosity of natural tears depends on shear-dependent reversible aggregation of tear proteins and lipids (unpublished personal communication). Of all the formulations I examined, DKSFM-HPMC, -Carbopol and -SH at concentrations of 0.2% and 0.4% as well as commercial Hypromellose® and Vislube® showed non-Newtonian behaviour. Shear-thinning or non-Newtonian behaviour is a property of solutions containing long polymeric macromolecules which are not strongly bonded into a globular form, such as HPMC, Carbopol and SH. The random orientation and interaction of these molecules produce high resistance to shear at low shear rate. At higher rates of shear, the molecules become aligned in the direction of the shearing force and offer much less resistance. Thus, solutions become less viscous, or more fluid, with increasing shear rate. Non-Newtonian fluids, with pseudoplastic behaviour, are more comfortable in the eye than Newtonian solutions of equivalent viscosity, since at high speed blinking non-Newtonian liquid exhibits less dragging effect on the ocular surface.

The shear rate associated with normal blinking has been estimated to range from 0 to > 40.000 s^{-1} (Doane 1980) and some formulations may tend to show non-Newtonian behaviour at much higher shear rates in eyes. Although I could only measure the viscosity

of the DKSFM-variations at the low-shear plateau, from 2 to 128 s^{-1}, due to the limitation of the viscometer, from these data 0.2% and 0.4% DKSFM-HPMC and 0.4% DKSFM-SH seem be suitable to use as tear substitutes with moderate viscosity and shear-thinning property.

4.3 THE STABILITY OF DKSFM-VARIATIONS

Chemical and physical stability are two of the essential qualities of drugs. A long shelf life of drugs is advantageous for commercial purpose since it demonstrates economic value to both manufacturers and consumers. According to the manufacturer DKSFM has a shelf-life of at least 3 months when stored at 2-4°C. I examined the chemical and physical properties of the self prepared DKSFM-variations after 3 months' storage at 4°C. Dry eye patients may keep a dropper bottle more than one month and any toxicity after long term storage needed to be excluded. Therefore, I also tested the ability of stored DKSFM-variations to support cell growth after 3 months of storage.

All solutions other than DKSFM-SH retested after 3 months of storage remained clear and without precipitates or any microscopic signs of microbial contamination. Precipitates had been observed for hyaluronate at the concentration of 0.1 and 0.2% after about 1 month and was obvious under the phase contrast microscope after a 24 hour incubation, while no contamination was observed with DKSFM-hyaluronate 0.4%. The pH and osmolarity of all the solutions maintained stable. The viscosity of 0.4% DKSFM-Carbopol increased and 0.4% DKSFM-SH decreased, although this did not influence cell proliferation. While the viscosity and effect on cell proliferation of fresh and stored DKSFM-HPMC remained unchanged. Also, Helin-Tanninen reported that 1% HPMC solution was microbiologically stable for 6 months and physically stable for 12 months (Helin-Tanninen, Naaranlahti et al. 2001). Moreover, HPMC has other potential superior advantages, such as easy availability, ease of preparation, storage at room temperature, ability to withstand autoclaving, and low cost.

Carbopol is a synthetic compound comprised of a cross-linked polymer of acrylic acid with a high molecular weight. Carbopol is a highly water and polar solvent soluble polymer, but tends to form clumps of particles, which prevent complete wetting of the interior polymer

particles and might cause mechanical irritation if used in the eye. To avoid this, Carbopol was added slowly and carefully to the DKSFM while constantly being stirred, using a low agitation of 500 rpm to prevent degrading the ingredients in DKSFM. However, the DKSFM-Carbopol preparation could still contain some half-hydrated polymers which might have become completely hydrated during the subsequent storage, thus further raising the viscosity of the solution. Another hypothesis to explain my findings is that the acrylic acid polymer might have cross-linked with DKSFM to form polymers with even higher molecular weight.

Hyaluronate is a long and complex molecule and has a flexible, open-coil conformation with ball shape, which resists compression. It is made up of a repetitive sequence of two modified simple sugars, glucuronic acid and N-acetylglucosamine. The decrease of viscosity of DKSFM-SH over 3 months may be due to its degradation over time, since SH molecules easily break down into smaller pieces and which then are no longer effective in keeping a ball shape. This will lead to a loss of lubricating ability, resistance to flow and a reduced viscosity.

4.2 NUTRIENT PROPERTIES OF DKSFM-VARIATIONS

Although increasing the viscosity enhances the residence time, this could at the same time reduce the epitheliotrophic properties of the medium as well as induce toxicity by potentially prolonging the contact of hazardous components with the ocular surface epithelia or by initiating interactions of some of the components. Therefore, in order to provide the minimal toxic substitute, an *in vitro* testing of the modifications of DKSFM was mandatory and was performed in five different culture models of ocular surface epithelia. DKSFM-HPMC, -Carbopol, -SH and -allantoin, as well as -fibronectin were tested for their support of cell viability, with DKSFM as positive control and 1 % BAC as negative control. BAC is a well known preservative and its toxicity on corneal and conjunctival epithelial cells has been described (Pfister and Burstein 1976). Commercial artificial tears containing the equivalent physically active component were also tested for comparison. The test exposure time ranged from 24 h to 144 h.

In KCS, tear deficiency often induces ocular surface epithelial disorders, including punctate keratitis and corneal ulceration (Rivas, Oroza et al. 1992). The maintenance of the corneal epithelial tight junctions is essential for the corneal barrier function. This is dependent in part on the ability of the corneal epithelium to undergo continuous renewal. The rate of renewal is dependent on a highly integrated balance between the processes of corneal epithelial proliferation, adhesion, migration, differentiation, and cell death. Briefly, there are three processes after corneal epithelium injury. First, corneal epithelial cells begin to migrate over the denuded area. When a monolayer of epithelial cells covers the denuded area, they begin to proliferate and the total thickness of the epithelium increases. Finally, the cells undergo differentiation and the epithelium returns to its well-differentiated, stratified structure as is observed in the normal uninjured corneal epithelium (Lu, Reinach et al. 2001). Since cell migration is the initial and essential process to obtain the integrity of corneal surface, I also examined the effect of DKSFM-variations on the migration of one corneal (CEPI-17-CL4) and one conjunctival (Chang) cell line with a colony-dispersion-assay.

4.2.1 DKSFM-HPMC

Methylcellulose (Methocel®) was reported in 1977 to be useful for lubricating intraocular lens implants during surgery prior to placement in the eye (Fechner 1977). It has long been used in ophthalmic surgery, gonioscopy and as a base for eyedrops. The active ingredient is HPMC, which is synthesized from methylcellulose, but has a higher hydrophilicity than methylcellulose because it consists of methoxy and hydroxypropyl side chains instead of hydroxyl in methylcellulose. This cellulose component that is widely distributed in nature, such as cotton and wood, is not a natural component of animals or humans. HPMC has been reported by several investigators to be safe and effective in cataract surgery (Glasser, Matsuda et al. 1986) (Kerr Muir, Sherrard et al. 1987) (Rosen, Brooks et al. 1987) and for use as a tear substitute (Dimoski, Latinovic et al. 1992; Toda, Shinozaki et al. 1996).

In the ATP and Calcein AM/EthD-1 assays, DKSFM-HPMC showed little effect on cell viability up to 72 hours treatment, though a slight decrease of cellular ATP levels was observed after 144 hours. DKSFM-HPMC maintained cellular viability better than DKSFM-allantoin, -Carbopol and -SH. This is contradictory to the results of Debbasch et al who showed that HPMC decreased the cell viability compared to SH and carbomer

(Debbasch, Pisella et al. 2000). The cytotoxicity response to HPMC may depend on how it is prepared. Purified HPMC free of protein substances and particulate debris has a lower toxicity for living tissues (Bittner 1998). The HPMC-product I used is highly purified, with a minimum of 94.5% HPMC which may explain why DKSFM-HPMC induced little to no toxicity even after long term exposure.

I observed a notable difference of cell proliferation between DKSFM-HPMC and commercial Hypromellose®. Although unpreserved Hypromellose® (0.3% HPMC) led to nearly 100% cell death at 24 hours, DKSFM-HPMC at concentration of 0.2% and even 0.4% still supported 100% cell growth. This can not be attributed to the physical properties, since the viscosity, surface tension and osmolarity of Hypromellose® and 0.4% DKSFM-HPMC are very similar. Toxicity of incubation with Hypromellose® might be due to its high pH (= 7.8), compared to DKSFM-HPMC (= 7.2), but it is more likely that this is a result of the complete lack of nutrients in Hypromellose®. Even when the Hypromellose® was highly diluted with DKSFM it reduced the ATP content of conjunctival epithelial cell cultures significantly than DKSFM-HPMC with the same dilution. This suggests a toxic effect of Hypromellose® components, although we used the unpreserved formulation of this commercially available tear substitute.

CEPI-17-CL4 cell migration was improved by 0.2% and 0.4% DKSFM-HPMC over 144 hours. To my knowledge, there is no report and no theoretical explanation why HPMC should stimulate cell migration. Again it is more likely that the cytokines in DKSFM may be responsible for the enhancement of migration, since EGF and FGF supported proliferation and migration of corneal epithelial cells (Grant, Khaw et al. 1992) (Imanishi, Kamiyama et al. 2000).

Varying concentrations of HPMC are used in current commercial tear formulations and 0.3% is the most popular one (Snibson, Greaves et al. 1992; Debbasch, Pisella et al. 2000; Iester, Orsoni et al. 2000). However, 0.5% HPMC has also been shown to be effective for the treatment of severe dry eye (Toda, Shinozaki et al. 1996). In my experiments the concentrations of 0.2% and 0.4% of HPMC in DKSFM resulted in a moderately viscous solution with well preserved supportive action on corneal and conjunctival epithelial cell migration and proliferation.

4.2.2 DKSFM-Carbopol

Carbopol polymers have long been used as rheology modifiers, suspending agents and stabilizers, in hair care, skin care, sun care, and other applications. Carbopol is also used in artificial tears because of its mucoadhesive property. Series of Carbopol products are available from Noveon Inc., including Carbopol 910, 940, 950 and 980. Although the type of Carbopol does not influence the mucoadhesive capacity (Ceulemans and Ludwig 2002), Carbopols with a higher numeric label have a higher purity with lower residual solvents. While Carbopol 940 is currently widely used in tear substitutes, such as Viscotears®, it contains benzene, which is a known carcinogen and hematopoietic toxicant (Faiola, Fuller et al. 2004) (Corti and Snyder 1998). However, Carbopol 980, which was used in the study presented here, is dissolved with ethyl acetate and cyclohexane instead.

Dose- and time-response ATP assays showed toxicity of 0.2% and 0.4% DKSFM-Carbopol, but not at lower concentrations. DKSFM-Carbopol and commercial Thilo-Tears® had different effect on cell proliferation, regardless of being diluted in DKSFM to the same concentrations of carbomer. 0.4% DKSFM-Carbopol had viscosity of 205 mPa.sec at shear rate of 1.7 s^{-1} and Thilo-Tears® even higher, since it was beyond the measuring range of the viscometer. Oechsner et al. reported that Thilo-Tears® had viscosity of 13,455 mPa.sec at 1 s^{-1} shear rate (Oechsner and Keipert 1999). The obvious cell damage caused by Thilo-Tears® might be due to its extremely high viscosity and the complete lack of nutrients in it. CEPI-17-CL4 cell migration stimulated by 0.05% DKSFM-Carbopol over 144 hours may also be explained by the growth factors such as EGF and FGF existing in DKSFM (Grant, Khaw et al. 1992) (Imanishi, Kamiyama et al. 2000).

Wilson et al have shown that carbomer based gels have a significantly extended ocular surface residence time compared to saline control (Wilson, Zhu et al. 1998). Carbomer was also reported to be effective and safe in the treatment of dry eyes, but blurred vision is the most common adverse event (Marner, Mooller et al. 1996). 0.2% carbomer reduced symptoms (gritty or foreign body sensation, burning sensation, dry eye sensation,

photophobia) and signs (conjunctival hyperaemia, ciliary injection, corneal and conjunctival epithelial staining) significantly better than polyvinyalcohol (PVA) in dry eyes (Brodwall, Alme et al. 1997; Bron, Mangat et al. 1998). Diebold reported that 0.3% carbomer had a notable cytotoxic effect after incubation of 0.5 hour *in vitro* (Diebold, Herreras et al. 1998). The concentration of 0.3% is still the most popular concentration in carbomer containing formulations on market, including Thilo-Tears®, Viscotears®, Lacrivisc® and Gel-larmes®. However, based on my results DKSFM-Carbopol at 0.2% does not provide adequate viscosity, and Carbopol in DKSFM with higher concentrations is too viscous to be comfortable in the eye and can also induce cytotoxicity.

4.2.3 DKSFM-hyaluronate

Hyaluronate is the natural biologic lubricating and shock-absorbing molecule of the musculoskeletal system and the eye. SH is found in high concentrations in the vitreous and connective tissue of the trabecular meshwork and in low concentrations in the aqueous humor and on the endothelium (Kahan and Kahan 1960) (Swann and Constable 1972) (Laurent 1983; Laurent and Granath 1983). It stabilizes cells and tissues and plays an important role during embryonic development and growth. At cellular level, it has been implicated in cell-to-cell interactions, cell matrix adhesion, cell mobility, and extracellular organization. SH has long and extensively been used in several diverse areas, including ophthalmic surgery and artificial tears. It can also be used in drug delivery, orthopaedics and in wound healing (Tan, Johns et al. 1990).

DKSFM-SH at 0.1% and higher concentrations showed near 100% toxicity, i.e. cell damage in the ATP assay. DKSFM-SH did not stimulate cell migration at any concentration, although SH was shown to stimulate corneal epithelial cell proliferation (Miyazaki, Miyauchi et al. 1996) (Inoue and Katakami 1993) and to accelerate corneal epithelial cell migration *in vitro* (Gomes, Amankwah et al. 2004) (Miyauchi, Sugiyama et al. 1990) (Sugiyama, Miyauchi et al. 1991). Several studies showed that hyaluronate eyedrops at 0.2% and 0.3% enhance tear film stability (Snibson, Greaves et al. 1990; Snibson, Greaves et al. 1992) and was considered to be well tolerated and effective in reducing Rose Bengal staining of the ocular surface in dry eyes (Aragona, Papa et al. 2002) (Condon, McEwen et al. 1999) (Laflamme and Swieca 1988; Nelson and Farris 1988).

Since my results with DKSFM-SH deviated so obviously from the currently available *in vivo* and *in vitro* data. The toxicity of DKSFM-SH could have resulted from

1) a poor quality of the original SH supply,
2) the experimental production process,
3) interactions of the DKSFM with the SH or
4) the additional supplementation of DKSFM-SH with antibiotics.

As mentioned in 2.2 the antibiotic combination used as additional supplement to DKSFM-SH induces no toxicity at the concentration used in the experiments discussed here. As the SH was – as labelled by the manufacturer – only suitable for laboratory use, I repeated the ATP assay with Healon® (1.4% SH) as a source for hyaluronate, which is widely used in cataract surgery. This combination supported proliferation to a level that was similar to DKSFM-HPMC. Although I can not conclude which of the above factors is responsible for the toxicity observed with DKSFM-SH, it becomes obvious from this experiment that only pharmacy grade materials should be used if a DKSFM-variation is tested in a clinical trial.

SH has been extracted from a number of sources, including the dermis of rooster combs, umbilical cords, and cultures of streptococci. Although the highly purified SH from each of these sources has the same structure, the molecular weight can vary and is a critical factor in the physical properties. The SH used in this study is extracted from human umbilical cords with the protein content less than 1% (Fluka) and its molecular weight is given as 3-5.8 million daltons (Bitter and Muir 1962) (Goebeler, Kaufmann et al. 1996). Vislube® (TRB CHEMEDICA) contains 0.18% SH with the molecular weight of 1.2-1.4 million daltons and is produced by bacterial fermentation. Healon® (Pharmacia) contains 1.4% SH with the molecular weight of 5 million daltons, which is extracted from rooster combs. There is still disagreement as to which source of SH provides the most consistently refined material without protein by-products and purity may be an important factor in toxicity. This study showed that the commercial pure SH from Fluka can not be simply dissolved in DKSFM since this is not only a very unstable solution in which the SH easily degrades, as shown in the viscosity measurements, but also induces toxicity. DKSFM-SH also became frequently contaminated by microbes during the cell culture experiments. This problem persisted even after adding antibiotics – unlike as in other DKSFM-variations – and is most likely a result of the high affinity of bacteria to the glucose molecules of SH. Therefore, to

formulate an ideal DKSFM-SH demands considerable technology. DKSFM-Healon® might be an alternative for the treatment of KCS, with the stimulating effect on cell proliferation and migration, but it is very costly.

The concentrations of most SH eye drops for dry eyes range from 0.1% to 0.4% (Shimmura, Ono et al. 1995) (Yokoi, Komuro et al. 1997) (Avisar, Creter et al. 1997) (Aragona, Di Stefano et al. 2002) and the minimum concentration of SH to delay the breakup of the tear film is 0.1% (Hamano, Horimoto et al. 1996). Debbasch concluded 0.18% SH had no cytotoxicity and was better tolerated than 0.3% carbomer after 24 hours (Debbasch, De La Salle et al. 2002). Since I only tested the effect of DKSFM-Healon® on cell proliferation after short term exposure, the potential effect after longer exposures needs further investigations.

4.2.4 DKSFM-allantoin

Allantoin, a uric acid derivative, was found in fetal allantoic fluid, from which it derives its name. Allantoin was believed to stimulate cell proliferation and wound healing. It was observed a significant elevation of the allantoin in wound fluid from chronic leg ulcers compared to acute surgical wound fluid (James, Hughes et al. 2003). The 85% of chronic recurrent leg ulcers was healed with the treatment of Silver-Zinc-Allantoinate cream for an average of 10 weeks (Margraf and Covey 1977). It is also useful in the treatment of skin burn and aphthous by removing exudates and foreign material from the wound and hastening the growth of new healthy tissue (Garnick, Singh et al. 1998) (Sheker, Black et al. 1972). I supplemented DKSFM with allantoin to improve its proliferation property, but found that this had no influence on cell proliferation after short-term incubation (24h), but cell proliferation tended to decrease after 96 h. In contrast to this allantoin at a concentration of 0.05% significantly improved the migration of CEPI-17-CL4 cells compared to other DKSFM-variations except 0.2% DKSFM-HPMC showed a similar positive effect on migration over 144 h.

Compared to HPMC, allantoin did not show any superior biophysical properties in this study. Allantoin did not improve the viscosity or surface tension of DKSFM, thus could not enhance the stability of tear film. Moreover, DKSFM-HPMC was better tolerated by

cells than DKSFM-allantoin. Therefore, HPMC is a better choice as a supplement to DKSFM than allantoin for the formulation of a nutrient and lubricant tear substitute.

4.2.5 DKSFM-fibronectin

Fibronectin is a large dimeric glycoprotein and involved in many cellular processes, including tissue repair, embryogenesis, blood clotting, and cell migration/adhesion. Fibronectin exists in two main forms:

1) as an insoluble glycoprotein dimer that serves as a linker in the extracellular matrix, and
2) as a soluble disulphide linked dimer found in the plasma (plasma fibronectin).

The plasma form is synthesized by hepatocytes, and the extracellular matrix form is made by fibroblasts, chondrocytes, endothelial cells, macrophages, as well as certain epithelial cells. Cell adhesion to fibronectin is mediated by the central cell-binding domain of fibronectin through an RGD (Arg-Gly-Asp) sequence (Potts and Campbell 1994). It can be used as a thin coating on tissue-culture surfaces to promote attachment, spreading, and proliferation of a variety of cell types.

Fibronectin was used in this study as a nutrient additive to the DKSFM. It was obtained as lyophilized human plasma fibronectin from Sigma. DKSFM-fibronectin supported cell proliferation at all the concentrations examined after 24 hours. Fibronectin was reported to promote the proliferation of 3T3 cells and retinal endothelial cells *in vitro* (Wilson, Ljubimov et al. 2003) (Nuttelman, Mortisen et al. 2001). DKSFM-fibronectin did not support CEPI-17-CL14 cell migration when tested over 72 hours, whereas in other studies fibronectin in serum free medium was found to facilitate migration of cultured rabbit corneal epithelial cells expanding from corneal blocks (Watanabe, Nakagawa et al. 1987) (Nishida, Nakamura et al. 1990) or cell suspensions (Wang, Kamiyama et al. 1994).

Fibronectin's principal function appears to be in cellular migration during wound healing and development (Mooradian, McCarthy et al. 1993) (Maldonado and Furcht 1995). Fibronectin is present in the tear film (Fukuda, Fullard et al. 1996) and found to stimulate attachment of corneal epithelial cells. It not only has haptotacic, but also chemotactic and chemokinetic activities for the corneal epithelial cells (Watanabe, Nakagawa et al. 1988). A number of investigators have used fibronectin eyedrops in a clinical study to heal

persistent corneal epithelial defects (PED) (Nishida, Ohashi et al. 1983; Boisjoly and Beaulieu 1991; Kim, Oh et al. 1992; McCulley, Horowitz et al. 1993; Gordon, Johnson et al. 1995), herpetic keratitis (Nishida, Nakagawa et al. 1985) and epithelial disorders in diabetic patients (Spigelman, Deutsch et al. 1987). Nelson and Gordon also used fibronectin in dry eyes, but did not find it more effective than commercial artificial tears, Liquifilm Tears®, containing 1.4% polyvinyl alcohol (Nelson and Gordon 1992). While Gordon who used lyophilized plasma fibronectin was unable to show its efficacy in PEDs (Gordon, Johnson et al. 1995), other authors used fibronectin directly purified from autologous plasma and showed improved wound healing of PEDs. For this *in vitro* study I had to use commercial lyophilized plasma fibronectin which did not support cell migration. This supports the importance of the way fibronectin is prepared and obtained on its cellular function and efficacy. Furthermore, some studies have shown the importance of a close interaction of epithelial cells with fibroblasts in corneal wound healing (Li and Tseng 1995) (Li and Tseng 1996). Since I used epithelial cell monocultures, this might also explain why DKSFM-fibronectin could not induce cell migration in my *in vitro* model.

4.3 COMPARISON OF CELL VIABILITY TEST SYSTEMS

Two cell viability assays based on different principles, the luminescence based ATP assay and the fluorescence based Calcein AM/EthD-1 assay, were compared in this study. In the ATP assay, cell viability is defined as the total amount of ATP of cells. ATP has been used as a tool for the functional integrity of living cells since ATP serves as the principal immediate donor of free energy and is present in all metabolically active cells (Petty, Sutherland et al. 1995). In the Calcein AM/EthD-1 assay, cell viability is defined as cell membrane permeability and intracellular esterase activity.

Both, the ATP and Calcein AM/EthD-1 assay, are very sensitive and reliable. The luminescence based ATP assay has been found to be more sensitive and reproducible than the traditional MTT assay (Petty, Sutherland et al. 1995). Its detection sensitivity is less than 20 cells (Lundin, Hasenson et al. 1986; Stanley 1986; Crouch, Kozlowski et al. 1993). Calcein AM is considered the optimal fluorescent dye, since this it is well retained in cellular targets and has a low pH sensitivity, bright fluorescence, reduced spontaneous leakage, and is not transferred among cells (Wang, Terasaki et al. 1993; Lichtenfels,

Biddison et al. 1994). The Calcein AM/EthD-1 assay is applicable to resting as well as activated human effector cells and different human cell lines (Neri, Mariani et al. 2001). Both assays require only half an hour to generate a measurable signal and therefore are more convenient than assays that require several hours of incubation. In addition, rapid assays reduce the chance of artifacts caused by interaction of test compounds with assay chemistry. They are performed in 96-well plates and give more than one set of data from different samples at the same time, which contributes to saving time and effort.

The ATP-assay can be used to assess proliferation as well as viability / toxicity, which - for this purpose - was defined as reducing the viability of cultured IOBA-NHC cells. I therefor compared which of the two assays, the ATP- or the Calcein AM/EthD-1-assay, is more sensitive in detecting viability / toxicity. The results confirmed that the ATP and the Calcein AM/EthD-1 assay correlated well in detecting toxicity in cells cultured with DKSFM-variations and DKSFM-commercial artificial tears for 24 hours. However, the ATP assay was more sensitive than the Calcein AM/EthD-1 in detecting toxicity at the concentration of 0.2% DKSFM-variations, which is a commonly used concentration of lubricant solutions.

Since both assays were performed in 96-well plates, but with different numbers of cells per well, they can not be compared directly. The ATP-assay was performed to determine proliferation and since contact inhibition in confluent cultures reduces proliferation (Partlow, Hanson et al. 1982) (Wieser and Oesch 1986), the cells were seeded at a lower density and test substance exposure started with non-confluent (30%) cell cultures. The Calcein-AM/EthD-1 assay was performed to assess toxicity, which *in vivo* is reduced since confluent cell layers cover the ocular surface and these are more resistant to toxicity than single cell (Wataha, Hanks et al. 1993) (Riss and Moravec 2004). These different test designs may have reduced the sensitivity of the Calcein-AM/EthD-1 assay. However, Calcein AM assay maintains the same sensitivity with 10^4/mL or 10-fold-lower dilution target tumour cells (Neri, Mariani et al. 2001). Moreover, Cree et al showed that variations in cell number per well do not influence the sensitivity of the ATP assay to detect toxicity and it therefor might be argued that this assay offers superior test conditions (Cree, Pazzagli et al. 1995).

4.4 COMPARISON OF DIFFERENT CELL CULTURE MODELS

In vitro systems offer the possibility of rapidly studying the influence of metabolites, mediators, or drugs on the behaviour of living cells in a controlled environment. Although they lack some modulating mechanisms such as neural pathways that are important in epithelial integrity in the long term, well-characterized human cell line cultures known to retain the features of the original tissue are often found to yield reproducible results.

This study was not only designed to formulate a nutrient lubricant but also aimed compare a number of human cell lines in order to establish whether the responses of cell lines to potentially toxic substances varies and which best reflects the sensitivity of primary cells. It also allowed the direct comparison between cell lines of corneal and conjunctival origin, since two human corneal (HCE-T and CEPI-17-CL) and two conjunctival epithelial cell lines (Chang and IOBA-NHC) were used.

These cell lines are all well characterised and have previously been shown to retain the specific features of their tissue of origin, i.e. corneal or conjunctival epithelium (Araki-Sasaki, Ohashi et al. 1995) (Sharif, Wiernas et al. 1998) (Offord, Sharif et al. 1999) (Bourcier, De Saint-Jean et al. 2000) (Chang 1954) (Diebold, Calonge et al. 2003). However, to validate the findings of my cell line experiments, I performed the proliferation assay also with primary rabbit corneal epithelial (RCE) cells. Primary rabbit corneal epithelium rather than human cells were cultured because of the limited human corneal donor material and the difficulties to culture epithelial cells from organ cultured donor tissue, which at the time of my experiments, was the only potential source of human corneal tissue at the University of Lübeck. Primary RCE cells were identified as corneal epithelial cells with the typical cobblestone morphology which was also observed in all cell lines. In addition, RCE cells showed positive with immunostaining for CK3 and negativity for CK19. This reflects the specific pattern of normal ocular surface epithelia, where the immunophenotypic definition of corneal epithelium is CK3+/CK19- and of conjunctival epithelium is CK3-/CK19+ (Chaloin-Dufau, Pavitt et al. 1993).

In the *dose-response proliferation experiments* over 24 hours which were quantified with the ATP assay, two corneal epithelial cell cultures, RCE and CEPI-17-CL4 cells, were more susceptible to toxicity than HCE, Chang, IOBA-NHC. However, all cell line models

agreed in general on the ranking of the toxicity of the test solutions. HCE-T and IOBA-NHC cells also showed no significant different results in the Calcein AM/EthD-1 assay. In *time-response experiments* a continuous exposure to the test solutions of up to 144 hours was also performed to detect potential low grade toxicity after long term incubation. This in fact tended to reveal toxicity for the test substances since the increasing cell damage was obviously observed when cells were incubated with DKSFM-Carbopol at concentrations higher than 0.1% from 24 h to144 h. DKSFM-HPMC at concentration of 0.4% exhibited toxicity after 72 h incubation but not before and this was better shown by CEPI-17-CL4 cells than other cell lines. To speculate what caused the observed albeit small differences in susceptibility among the cell lines is beyond the scope of this thesis. However, based on my results the viability / proliferation of primary corneal epithelial cells is best represented by the CEPI-17-CL4 cell line. If no source of primary corneal epithelial cell material is available, this cell line is recommended for use in future studies.

Migration was assessed in a colony dispersion assay. In this assay Chang cells despite remaining viable failed to migrate at all, while CEPI-17-CL4 cells did migrate when incubated with DKSFM-variations that had little or no toxic effects. This was not assessed by previous authors using these cell lines. I can not explain these findings, since other authors found that HCE-T cells migrate easily with EGF or Nerve Growth Factor (NGF) (You, Ebner et al. 2001) and DKSFM includes at least one of these factors. Due to the lack of sufficient supply of RCE cells the migration response of cell lines could not be verified with primary cells. However, You et al also showed that the migration of primary corneal epithelial cells in EGF and NGF is comparable with HCE-T cells (You, Ebner et al. 2001).

The detection for sensitivity of cell culture toxicity studies not only varies with cell type and cell line, but also with other factors such as the plate format (van Wyk, Olivier et al. 2001) (Yamamoto, Honma et al. 1999). It should also be noticed that some variations among replicate samples is caused by the cells, since at least cultured primary cells exist as a heterogeneous population (Machida, Spangenburg et al. 2004). Hence the data from most plate-based assay formats represent an average of the signal from the population of cells. In this study, the coefficient of variation of positive controls for the experiments performed with the four different cell lines and the primary cells was under 20%, which reflects the genetic and phenotypic heterogeneity of normal tissue (Cree and Andreotti 1997). In addition, all experiments were performed in triplicates and repeated at least once. Since

this repetition always confirmed the findings of the first set of experiments, my results can be considered to be reproducible.

Redundancy in my experiments was achieved not only by using five different models, but also by assessing the effect of DKSFM-variations in three different assay types. To perform such an extensive experimental program with a large variety of DKSFM-variations can realistically only be achieved *in vitro.* Although the complex physical and molecular interactions of tear film and ocular surface *in vivo* can not be reproduced completely *in vitro*, the obviously very similar results found in these different models suggest that my results are likely to reflect the behaviour of primary human corneal and conjunctival cells.

5 CONCLUSION

In conclusion, the answers to the scientific questions raised in this thesis are as follows:

(1) What are the biochemical and biophysical properties of DKSFM and the DKSFM-variations produced?

Supplementing DKSFM with viscosity enhancing substances changed the pH apparently only when Carbopol was used, which required extensive adjustment to physiological levels until the process of dissolving was completed after 24 hours. Osmolarity remained in the isotonic range, except if fibronectin was added at a concentration of 0.005% or higher. The viscosities of DKSFM-HPMC, -Carbopol and -SH at all the concentrations examined were significantly higher than DKSFM. Both, DKSFM-HPMC at 0.2% to 0.4% and DKSFM-SH at 0.4% showed moderate viscosity and non-Newtonian behaviour. The surface tension of DKSFM-HPMC was around 50 mN/m, which is closer to human tears than that of DKSFM, DKSFM-Carbopol and -SH, which had a surface tension of about 70 mN/m. DKSFM-HPMC remained stable for up to 3 months when stored at 4°C, while the viscosity of DKSFM-Carbopol and -SH changed significantly.

(2) Does supplementation of DKSFM with pharmacy grade single compounds or ready made pharmaceutical tear drops reduce or improve its nutrient properties?

The nutrient properties of DKSFM-variations depended on the type and concentration of supplements used. DKSFM with HPMC supported migration well and did not induce toxicity at 72 h, but only reduced relative cell growth by up to 25% at 144 h at any concentration tested. Fibronectin did not alter the relative cell growth, but was unable to support cell migration. Adding Carbopol, SH or allantoin to DKSFM induced dose and time-dependent toxicity and DKSFM-Carbopol and allantoin could maintain cell migration. DKSFM-Carbopol at ≥ 0.2% induced 90% cell death within 24 h and reduced relative cell growth from 24 h on. Contrary to previous reports SH – at least when mixed with DKSFM - reduced cell growth at ≥ 0.2% from 24 h on and induced cell death at 0.4% at 24 h. DKSFM-allantoin at concentration of 0.4% also reduced relative cell growth

within 24 h but did not induce cell death even at 72 h. The cell growth supporting ability of DKSFM was better when single compounds were used as supplements, than with ready made pharmaceutical products, except for hyaluronate. Differences in the production protocols of the test-solutions are thus likely to be responsible for some of the findings in this study contradicting other in-vitro results.

The ranking of the cell growth supporting ability of the DKSFM-variations was DKSFM-HPMC > -allantoin > -fibronectin > -Carbopol > -hyaluronate. This ranking was identical for all 4 cell lines used as test cultures. The ranking of cell migration support was DKSFM-HPMC > -allantoin > -Carbopol- > -SH /-fibronectin.

(3) Which of the two quantitative assays detects cytotoxicity at the lower concentration of the test substances?

The results of the ATP assay and the Calcein AM/EthD-1 assay correlated well for cells treated with DKSFM-variations for 24 h at lower concentrations. However, the ATP assay appeared to be more sensitive in detecting cytotoxicity of DKSFM-variations ≥ 0.2%.

(4) Do various ocular epithelial cell lines show a similar response, in terms of support of proliferation upon exposure to the substances tested and how does this response compare to primary epithelial cells?

HCE-T, Chang and IOBA-NHC cells could be equally used to study cytotoxicity of DKSFM-variations in-vitro. These 3 cell lines – although of different origin - showed no significant difference for proliferation in the ATP assay. However, primary rabbit corneal epithelial cells and CEPI-17-CL4 cells showed both an increased and equal susceptibility and hence the latter cell line might be preferable for future testing.

In summary my results suggest that DKSFM supplemented with HPMC at 0.2% or 0.4% can be used to improve its lubrication and nutrition for corneal and conjunctival epithelial cells. Its retention time, as well as efficacy to improve subjective symptoms and objective signs of ocular surface disease should be tested in a clinical randomized controlled trial.

6 SUMMARY

Introduction: The tear film has nutrient and lubricant properties. In dry eyes the ocular surface is compromised due to a lack of lubrication and nutrition. The ideal artificial tear drops should therefor substitute both components, however, the currently available products are primarily optimised for their lubricant properties. Cell culture media specifically designed for epithelial cells are now available as fully defined products and could potentially be used in eyedrops to provide nutrition to the ocular surface. However, since their lubricant properties are unknown and likely to require adjustment to increase the residence time and stability of the substitute tear film in order to provide sufficient mechanical protection and comfort for the ocular surface. As dry eye sufferers often use such medications several times per day, it is also essential to minimize any potential toxicity of the product. I therefor aimed to produce a novel tear substitute capable of providing lubrication and nutrition in one, based on a defined keratinocyte serum free cell culture medium, DKSFM, supplemented with various viscous and nutrient agents. In addition I also aimed to compare the sensitivity of 4 different cell lines of ocular surface origin and two *in vitro* assays to assess cell viability.

Materials and Methods: Viscous supplements included hydroxypropylmethylcellulose (HPMC), Carbopol, and sodium hyaluronate (SH) and nutrient additive substances were fibronectin and allantoin. To exclude that any toxicity observed in this study was due to the self-production, commercially available ophthalmic products, i.e. Hypromellose® (0.3% HPMC), Thilo-Tears® (0.3% carbomer), Vislube® (0.18% SH) and Healon® GV, were used as control supplements. These were added as single compounds to DKSFM at a concentration of 0.0001% to 0.4%. The pH and osmolarity of all the formulations were controlled and adjusted to be within the physiological range. The biophysical properties, i.e. viscosity and surface tension, were determined with a rheometer and an electronic manometer. Two human corneal epithelial cell lines (HCE-T and CEPI-17-CL4) and two human conjunctival epithelial cell lines (Chang and IOBA-NHC) as well as primary rabbit corneal epithelial cells were used to investigate cell proliferation, viability and migration in response to the formulations by means of a luminescence based ATP-assay, a Calcein AM/EthD-1 assay and a colony dispersion assay. All solutions were stored for 3 months at 4°C and retested to assess stability.

Results: The viscosity of DKSFM was around 0.75 mPa.sec at shear rates of 1.7-128.5 s^{-1}. HPMC, Carbopol and SH increased the viscosity of the DKSFM significantly at all the concentrations, but only 0.2% and 0.4% DKSFM-HPMC and 0.4% DKSFM-SH showed both moderate viscosity and non-Newtonian behaviour. However, only the viscosity of DKSFM-HPMC remained stable for up to 3 months when stored at 4°C, while the viscosity of DKSFM-Carbopol and -SH changed significantly. The surface tension of DKSFM, DKSFM-Carbopol and -SH was around 70 mN/m and thus similar to water. Only DKSFM-HPMC showed a surface tension similar to tears of about 50 mN/m.

The ranking of the cell growth supporting ability of the DKSFM-variations was DKSFM-HPMC > -allantoin > -fibronectin > -Carbopol > -hyaluronate. This ranking was identical for all 4 cell lines used. The ranking of cell migration support was DKSFM-HPMC > -allantoin > -Carbopol- > -SH /-fibronectin. The proliferation supporting ability of DKSFM-variations was better when single compounds were used as supplements than ready made pharmaceutical products, except for hyaluronate.

The ATP-response curves of the human corneal epithelial cell line CEPI-17-CL4 was closest to the reaction of primary rabbit corneal epithelial cells and both were more sensitive to toxicity than the other three cell lines. The ATP assay was more sensitive in detecting toxicity than the Calcein AM/EthD-1 assay.

Conclusion: The human corneal epithelial cell line, CEPI-17-CL, and the luminescence based ATP assay, are preferable for future testing. DKSFM-HPMC at 0.2 or 0.4% showed – of all solutions tested - superior lubricant and nutrient properties and good stability *in vitro*. Its retention time in the eye and effect on signs and symptoms of dry eyes should be tested in a clinical, randomised, controlled trial *in vivo*.

7 ZUSAMMENFASSUNG

Einleitung: Der Tränenfilm hat ernährende und benetzende Eigenschaften. Bei trockenem Auge ist die Augenoberfläche aufgrund der fehlenden Benetzung und Ernährung oft beeinträchtigt. Das ideale Tränenersatzmittel sollte daher beide Komponenten substituieren. Die gegenwärtig erhältlichen Produkte sind jedoch primär bezüglich ihrer benetzenden Eigenschaften optimiert. Mittlerweile sind speziell für Epithelzellen entwickelte und vollständig definierte Zellkulturmedien erhältlich, die potentiell als Augentropfen zur Ernährung der Augenoberfläche verwendet werden könnten. Deren Benetzungseigenschaften sind jedoch unbekannt und müssen für den klinischen Einsatz sicherlich optimiert werden, um die Verweildauer auf dem Auge und damit die Stabilität und Verträglichkeit des Ersatztränenfilms zu gewährleisten. Da Patienten mit trockenem Auge z. T. sehr häufig Tränenersatzmittel applizieren, muß die Toxizität eines künstlichen Tränenersatzmittels möglichst gering sein. Mein Ziel war es daher, basierend auf einem definierten serum-freien Medium zur Zellkultur von Keratinozyten ein neues Tränenersatzmittel durch Zusatz von benetzenden und nutritiven Mitteln zu entwickeln, dass sowohl benetzende als auch ernährende Eigenschaften hat. Zusätzlich galt es, die Sensitivität von 4 verschiedenen Zelllinien von Augenoberflächenepithelien sowie von 2 Toxizitätsassays experimentell zu vergleichen.

Material und Methoden: Als viskositätsteigernde Zusätze verwandte ich Hydroxypropylmethylcellulose (HPMC), Carbopol und Natriumhyaluronat (SH). Als nutritive Zusätze wurden Fibronectin oder Allantoin getestet. Um auszuschließen, dass ein eventuell beobachteter Effekt nur Folge der Eigenherstellung der Zusatzlösungen war, testete ich zusätzlich kommerziell erhältliche, in der Ophthalmologie verwendete Produkte mit gleichem Inhaltsstoff [Hypromellose® (0.3% HPMC), Thilo-Tears® (0.3% Carbomer) und Vislube® (0.18% SH), Healon® GV] als Kontrolllösungen. Alle Lösungen wurden als Einzelkomponenten dem DKSFM in Konzentrationen von 0.0001% to 0.4% zugesetzt. Der pH und die Osmolarität aller Testlösungen wurde kontrolliert und ggf. auf physiologische Werte justiert. Die biophysikalischen Eigenschaften, d. h. die Scheerraten-abhängige Viskosität und die Oberflächenspannung, wurden mit einem Rheometer und einem elektronischen Manometer bestimmt. Bei zwei humanen Hornhautepithelzelllinien (HCE-T und CEPI-17-CL4), zwei humanen Bindehautepithelzelllinien (Chang and IOBA-NHC) sowie primären Hornhautepithelzellen vom Kaninchen wurde die Zellproliferation, -

vitalität und Migration nach Inkubation mit den DKSFM-Varianten mit Hilfe eines lumineszenz-basierten ATP-Assay, einer Doppelfluoreszenzfärbung (Calcein AM/EthD-1 Assay) und eines Kolonie-Dispersionsassays bestimmt. Alle Lösungen wurden für 3 Monate bei 4°C gelagert und erneut getestet.

Ergebnisse: Die Viskosität von DKSFM betrug 0.75 mPa.sec bei einer Scheerrate von 1.7-128.5 s^{-1}. HPMC, Carbopol und SH erhöhten die Viskosität von DKSFM signifikant in allen Konzentrationen, aber lediglich 0.2% und 0.4% DKSFM-HPMC und 0.4% DKSFM-SH zeigten sowohl eine mäßige Viskosität als auch ein Nicht-Newton'sches Verhalten. Nach 3 Monaten Lagerung bei 4°C war lediglich die Viskosität von DKSFM-HPMC stabil geblieben. Die Oberflächenspannung von DKSFM, DKSFM-Carbopol und -SH betrug etwa 70 mN/m und war damit vergleichbar der von Wasser. Allein DKSFM-HPMC zeigte einen tränenähnlichen Wert von etwa 50 mN/m.

Der proliferationsfördernde Effekt der DKSFM-Varianten nahm in folgender Reihe ab: DKSFM-HPMC > -Allantoin > -Fibronectin > -Carbopol > -Hyaluronat. Diese Reihenfolge ergab sich für alle 4 verwendeten Zelllinien. Der migrationsfördernde Effekt nahm in folgender Reihe ab: DKSFM-HPMC > -Allantoin > -Carbopol- > -Hyaluronat /-Fibronectin. Die Zellproliferation wurde durch DKSFM, das mit Einzelzusatzstoffen ergänzt worden war, besser unterstützt als durch solche Mischungen, bei denen pharmazeutisch erhältliche Präparate als Substitute verwendet worden waren.

Die ATP-Kurven der humanen Hornhautepithelzelllinie CEPI-17-CL4 ähneltem der von primären Kaninchenepithelezellen am besten. Beide Zellkulturmodelle waren sensitiver als die anderen drei Zelllinien. Der ATP-Assay erwies sich als sensitiver im Nachweis von Zytotoxizität.

Schlußfolgerung:

Die humane Hornhautepithelzelllinie, CEPI-17-CL, und der lumineszenzbaiserte ATP-Assay sind für zukünftige Toxizitätsuntersuchungen in-vitro aufgrund der besseren Sensitivität zu bevorzugen. DKSFM-HPMC 0.2 oder 0.4% weist in-vitro von allen getesteten Lösungen die besten Benetzungs- und Ernährungseigenschaften auf und ist bei einer 3-monatigen Lagerung bei 4°C stabil. Seine Verweildauer auf dem Auge und seine Effektivität in der Therapie von Symptomen und Zeichen des trockenen Auges sollte in einer klinischen, randomisierten, kontrollierten Studie getestet werden.

8 ABBREVIATIONS

+	positive
-	negative
°C	degrees Celsius
%	percent
µL	microliter
µm	micrometer
ANOVA	analysis of variance
APAAP	alkaline phosphatase anti-alkaline phosphatase
ATP	adenosine triphosphate
AUC	areas under the curve
BAC	benzalkonium chloride
Ca	calcium
Calcein-AM	calcein-acetoxymethyl
cAMP	cyclic adenosine monophosphate
CG	cell growth
cGMP	cyclic guanosine monophosphate
Cl	chloride
CK	cytokeratin
CO_2	carboxic oxide
CV	Coefficient of Variation
CV	Cell Viability
DKSFM	defined keratinocyte serum free medium
ECM	extracellular matrix
EDTA	Ethylene Dinitro Tetraacetic Acid
EGF	epidermal growth factor
EthD-1	Ethidium-homodimer-1
FBS	fetal bovine serum
FGF	fibroblast growth factor
GS	growth supplement
g	gram
h	hour
HA	hyaluronic acid

HPMC	hypromellose, hydroxypropylmethylcellulose
IgA	immunoglobulin A
IgG	immunoglobulin G
IL	interleukin
K	potassium
KCS	keratoconjunctivitis sicca
KGF	keratinocyte growth medium
PAA	polyacrylic acid
PBS	phosphate buffer solution
PDGF	platelet-derived growth factor
Ps	Pascal.sec
M	mole
mEq	milliequivalent
mg	microgram
MI	maximum inhibition
min	minute
mM	micromole
MO	no inhibition
MTT	3-[4,5-dimethyl(thiazol-2-yl)-3,5-diphenyl] tetrazolium
Na	sodium
NaOH	sodium hydroxide
ng	nanogram
Osm	osmole
P	probability
PED	persistent epithelial defect
PVA	polyvinyalcohol
RCE	rabbit corneal epithelial
SD	standard deviation
SH	sodium hyaluronate
ST	surface tension
TBS	tris buffer solution
TGF	transforming growth factor
U	unit

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10 APPENDIX

Tab. 1: Components of DKSFM and viscoelastic control solutions

DKSFM and growth supplement

Trace elements, electrolyte
Sodium acetate, disodium phosphate dibasic, sodium chloride , sodium sulphate, potassium chloride, calcium chloride, magnesium chloride.

Hormones
Zinc insulin human, hydrocortisone.

Amino acid
L-alanine, glycine, L-arginine hydrochloride, L-asparagine, L-aspartic acid, L-glutamine.

Vitamines
Vitamine B12, ascorbic acid 2-phosphate, niacinamide, pyridoxine hydrochloride, riboflavin, thlamine hydrochloride.

Growth supplements
rEGF human and acidic FGF.

Hypromellose®
Hypromellose 400, sodium chloride, potassium chloride, borax, boric acid, disodium edentate and purified water.

Thilo-Tears®
Carbomer 974P, sorbitol, sodium hydroxide and purified water.

Vislube®
Sodium hyaluronate, sodium chloride, potassium chloride, disodium-hydrogenphosphate, sodium citrate, magnesium chloride, calcium chloride and water.

Healon®
Sodium hyaluronate 7000, sodium chloride, sodium dihydrogen phosphate, sodium monohydrgen phosphate and water.

Tab. 2: pH, osmolarity, surface tension (ST) of DKSFM, DKSFM-variations and commercial artificial tears

Substances	Concentrations (%)	PH	Osmolality (mmol/kg)	ST (pascals)
DKSFM		7,2	278	70,9
DKSFM-HPMC	0,0125	7,2	298	51,1
	0,025	7,2	304	48,5
	0,05	7,2	300	45,9
	0,1	7,2	291	51,8
	0,2	7,2	298	53,8
	0,4	7,2	290	53,5
DKSFM-Hypromellose®	0,0125	7,1	305	-
	0,025	7,1	308	-
	0,05	7,2	307	-
	0,1	7,3	307	-
	0,2	7,7	306	-
Hypromellose®	0,3	7,8	302	51,3
DKSFM-Carbopol	0,0125	7,2	279	71,4
	0,025	7,2	276	70,5
	0,05	7,2	277	70,8
	0,1	7,2	278	73,1
	0,2	7,2	284	75,3
	0,4	7,2	289	80,1
DKSFM-Thilo-Tears®	0,0125	7	308	-
	0,025	7	306	-
	0,05	7	307	-
	0,1	6,8	310	-
	0,2	6,7	319	-
Thilo-Tears®	0,3	7,1	329	73,1
DKSFM-SH	0,0125	7,2	290	70,7
	0,025	7,2	294	70,7
	0,05	7,2	277	70,3
	0,1	7,2	279	69,8
	0,2	7,2	282	69,5
	0,4	7,2	297	68,7
DKSFM-Vislube®	0,0125	7,1	294	-
	0,025	7,1	283	-
	0,05	7,1	264	-
	0,1	7	225	-
Vislube®	0,18	7,1	158	69,2
DKSFM-Healon®	0,0125	7,2	298	73,6
	0,025	7,2	299	72,9
	0,05	7,2	301	70,9
	0,1	7,2	301	71,4
	0,2	7,2	304	72,3
	0,4	7,2	304	72,6
DKSFM-allantoin	0,0125	7,3	302	69,7
	0,025	7,3	300	69,1
	0,05	7,3	288	70,3
	0,1	7,3	296	69,7
	0,2	7,3	305	69,5
	0,4	7,3	308	70,1
DKSFM-fibronectin	0,0001	7,3	299	-
	0,001	7,3	299	-
	0,005	7,3	340	-
	0,01	7,3	329	-
	0,1	7,3	569	67,8

Tab. 3: Viscosity of DKSFM, DKSFM-variations and commercial artificial tears

Tab. 3a

Shear rate	DKSFM	Concentrations of DKSFM-HPMC						Hypromellose®
(sec^{-1})		0,4%	0,2%	0,1%	0,05%	0,025%	0,0125%	
1,75	0,82	19,02	30,21	2,99	8,95	5,22	7,25	5,22
2,37	0,71	15,68	24,75	1,37	5,78	4,95	5,93	5,23
3,23	0,69	14,14	18,18	1,33	4,65	3,84	4,20	5,25
4,39	0,80	11,74	13,52	1,43	3,27	2,82	3,21	5,20
5,96	0,72	10,38	11,70	1,38	2,51	2,51	2,23	5,14
8,11	0,79	9,57	8,68	1,38	2,17	1,69	1,71	5,07
11,02	0,77	8,58	6,63	1,36	1,84	1,54	1,51	5,09
14,98	0,78	8,05	5,57	1,36	1,61	1,31	1,37	5,05
20,4	0,76	7,71	4,58	1,33	1,44	1,25	1,29	5,02
27,7	0,76	7,48	3,82	1,33	1,37	1,18	1,24	5,03
37,6	0,77	7,24	3,34	1,32	1,33	1,13	1,21	4,97
51,2	0,77	7,04	3,09	1,32	1,29	1,07	1,16	4,88
69,5	0,78	6,82	2,63	1,32	1,24	1,05	1,15	4,80
94,5	0,79	6,68	2,48	1,34	1,22	1,03	1,16	4,75
128,5	0,80	6,55	2,40	1,39	1,22	1,05	-	4,71

Tab. 3b

Shear rate	Concentrations of DKSFM-Carbopol					
(sec^{-1})	0,4%	0,2%	0,1%	0,05%	0,025%	0,0125%
1,75	205,37	3,59	1,64	1,12	0,90	0,82
2,37	175,74	3,51	1,65	1,04	0,99	0,82
3,23	149,48	3,47	1,58	1,01	0,89	0,81
4,39	128,54	3,47	1,57	1,01	0,83	0,80
5,96	110,49	3,44	1,55	0,99	0,90	0,79
8,11	94,87	3,41	1,53	0,98	0,84	0,80
11,02	81,99	3,38	1,53	0,98	0,85	0,80
14,98	71,29	3,35	1,52	0,98	0,84	0,80
20,4	61,80	3,36	1,50	0,97	0,84	0,80
27,7	54,07	3,30	1,50	0,97	0,84	0,80
37,6	47,28	3,26	1,50	0,96	0,84	0,81
51,2	41,60	3,23	1,50	0,97	0,84	0,81
69,5	36,77	3,17	1,50	0,97	0,85	0,82
94,5	32,57	3,12	1,52	0,98	0,86	0,82
128,5	17,78	3,07	1,52	0,98	0,87	0,84

Tab. 3c

Shear rate	Concentrations of DKSFM-SH						Vislube®
(sec^{-1})	0,4%	0,2%	0,1%	0,05%	0,025%	0,0125%	
1,75	24,99	4,93	2,32	1,49	1,20	1,12	7,46
2,37	25,03	4,83	2,25	1,48	1,15	1,10	7,15
3,23	24,85	4,93	2,18	1,45	1,17	1,01	7,27
4,39	24,96	4,84	2,26	1,46	1,13	1,01	7,28
5,96	24,92	4,86	2,19	1,45	1,12	0,96	7,32
8,11	24,76	4,89	2,22	1,43	1,11	0,98	7,32
11,02	24,80	4,87	2,22	1,43	1,10	0,97	7,28
14,98	24,71	4,87	2,22	1,43	1,11	0,96	7,31
20,4	24,51	4,86	2,21	1,42	1,09	0,95	7,23
27,7	24,38	4,86	2,19	1,41	1,09	0,94	7,22
37,6	24,07	4,84	2,20	1,41	1,09	0,94	7,19
51,2	23,76	4,82	2,21	1,41	1,09	0,94	7,15
69,5	23,26	4,80	2,20	1,40	1,09	0,94	7,11
94,5	22,56	4,77	2,19	1,41	1,10	0,95	7,07
128,5	21,79	-	2,19	1,42	1,12	0,96	6,99

Tab. 3d

Shear rate	Concentrations of DKSFM-allantoin						DKSFM-fibronectin
(sec^{-1})	0,4%	0,2%	0,1%	0,05%	0,025%	0,0125%	0,1%
1,75	1,12	1,20	0,75	0,37	0,82	0,75	0,97
2,37	0,66	1,04	1,15	0,93	0,82	0,78	0,99
3,23	0,89	0,93	0,97	0,57	0,77	0,79	0,97
4,39	0,83	0,89	0,74	0,83	0,77	0,65	0,92
5,96	0,79	0,81	0,94	0,77	0,77	0,74	0,92
8,11	0,77	0,79	0,80	0,74	0,77	0,71	0,88
11,02	0,79	0,76	0,77	0,76	0,76	0,75	0,89
14,98	0,78	0,77	0,74	0,76	0,76	0,75	1,02
20,4	0,76	0,76	0,76	0,76	0,75	0,74	0,91
27,7	0,76	0,76	0,77	0,75	0,75	0,78	0,90
37,6	0,76	0,76	0,77	0,76	0,75	0,78	0,90
51,2	0,77	0,76	0,78	0,77	0,76	0,78	0,88
69,5	0,77	0,77	0,78	0,77	0,76	0,79	0,88
94,5	0,78	0,77	0,79	0,78	0,77	0,80	0,88
128,5	0,79	0,78	0,80	0,79	0,78	0,79	0,89

Tab. 4: Dose- and time-response ATP assay for 4 cell lines treated with DKSFM-variations from 24 to 144 h. The values are presented as mean and CV of relative cell growth (%) for triplicate samples and represent relative ATP levels of cell cultures incubated with test solutions compared to negative and positive control cultures.

Tab. 4a: HCE-T

DKSFM-supplements	Time (h)		Concentrations (%)					
			0,0125	0,025	0,05	0,1	0,2	0,4
HPMC	**24**	mean	102,47	101,60	95,13	137,77	92,30	98,82
		CV	1,48	6,05	5,26	35,75	0,24	9,32
	48	mean	105,69	151,50	132,79	117,94	136,76	123,78
		CV	10,87	3,68	7,76	6,74	8,40	9,00
	72	mean	124,77	129,66	136,09	132,48	157,49	112,43
		CV	14,44	22,98	12,59	4,73	23,24	6,56
	96	mean	100,67	107,21	105,86	107,28	122,04	86,28
		CV	3,71	12,91	6,08	8,68	10,50	4,02
	144	mean	87,64	79,78	74,37	101,05	83,25	77,39
		CV	11,23	6,56	9,63	9,33	7,59	2,33
Carbopol	**24**	mean	116,03	113,32	99,62	81,33	42,58	24,23
		CV	3,78	5,50	1,49	3,76	1,33	1,89
	48	mean	127,38	127,56	144,34	98,29	53,64	9,38
		CV	9,40	22,13	13,86	10,02	5,46	4,02
	72	mean	97,63	94,40	73,82	40,67	6,62	1,05
		CV	13,73	2,80	1,74	4,59	0,55	0,21
	96	mean	71,05	78,03	56,44	29,41	5,16	1,83
		CV	6,93	5,63	3,60	2,18	0,26	0,29
	144	mean	58,87	53,96	47,62	24,28	1,34	0,02
		CV	5,50	1,20	5,61	2,15	0,07	0,01
SH	**24**	mean	112,67	124,46	108,27	-0,07	-0,15	-0,12
		CV	12,44	10,30	11,08	0,04	0,02	0,04
	48	mean	98,84	123,71	124,19	-0,02	0,37	-0,06
		SD	10,75	11,69	10,18	0,10	0,51	0,02
	72	mean	128,04	134,16	112,61	-0,07	-0,09	-0,08
		CV	1,57	7,73	7,05	0,01	0,01	0,01
	96	mean	83,75	97,35	94,25	72,35	-0,02	-0,14
		CV	1,26	4,91	1,33	4,16	0,09	0,07
	144	mean	83,95	85,15	84,75	84,64	0,08	-0,05
		CV	0,00	12,15	4,09	2,42	0,09	0,03
allantoin	**24**	mean	106,29	116,66	123,50	113,92	100,75	56,55
		CV	11,65	13,65	7,88	11,58	8,71	5,79
	48	mean	103,09	111,09	94,42	95,77	102,89	77,07
		CV	2,38	3,26	4,51	7,09	6,17	2,34
	72	mean	122,76	117,51	128,67	120,35	113,26	56,70
		CV	5,56	17,24	19,42	9,83	8,69	8,52
	96	mean	84,36	83,52	72,42	72,94	49,88	48,84
		CV	6,97	13,27	10,08	2,37	16,72	1,64
	144	mean	59,58	60,11	77,41	72,25	61,66	27,85
		CV	1,60	1,94	6,56	7,33	6,34	1,67

Tab. 4b: CEPI-17-CL4

DKSFM-supplements	Time (h)		concentrations (%)					
			0,0125	**0,025**	**0,05**	**0,1**	**0,2**	**0,4**
HPMC	**24**	mean	81,62	90,51	105,43	110,21	120,06	116,73
		CV	6,75	16,05	7,11	11,57	11,89	21,65
	48	mean	142,79	142,64	167,78	143,19	152,73	158,27
		CV	14,74	6,70	3,89	6,54	6,23	9,16
	72	mean	117,50	103,36	131,19	135,96	136,70	119,41
		CV	13,25	17,70	10,73	5,00	9,11	3,83
	96	mean	48,50	42,59	48,60	49,17	50,68	44,13
		CV	42,26	37,42	50,63	53,15	52,69	46,13
	144	mean	34,67	32,57	36,65	35,77	37,49	31,37
		CV	13,30	9,29	15,89	18,90	17,39	16,88
Carbopol	**24**	mean	30,08	26,42	34,39	35,94	35,86	31,46
		CV	10,62	10,63	12,36	12,11	12,52	10,34
	48	mean	95,50	116,39	84,92	27,73	9,67	12,91
		CV	13,60	18,90	4,78	2,38	1,50	0,74
	72	mean	32,45	140,44	107,71	36,44	5,98	0,90
		CV	2,83	0,60	5,25	4,31	0,19	0,41
	96	mean	110,08	89,76	102,18	51,19	5,71	-0,22
		CV	23,05	5,46	17,24	2,85	2,93	0,01
	144	mean	148,18	143,03	151,00	97,64	2,20	-0,21
		CV	4,77	16,64	14,50	8,65	0,42	0,02
SH	**24**	mean	114,30	104,79	79,50	-0,11	-0,48	-0,54
		CV	5,09	12,51	2,41	0,08	0,05	0,01
	48	mean	113,27	99,98	110,94	0,94	-0,38	-0,07
		SD	9,02	8,76	2,61	1,14	0,04	0,18
	72	mean	-	-	-	-	-	-
		CV	-	-	-	-	-	-
	96	mean	89,10	98,27	27,06	-0,17	-0,21	-0,25
		CV	0,17	8,97	4,81	0,04	0,05	0,03
	144	mean	108,70	149,75	43,27	0,03	-0,09	-0,19
		CV	7,73	15,58	4,08	0,04	0,05	0,04
allantoin	**24**	mean	49,87	88,88	83,02	91,41	88,81	93,60
		CV	3,72	7,79	7,45	6,95	2,97	5,53
	48	mean	106,20	112,06	94,95	94,58	97,14	71,92
		CV	9,03	11,50	5,87	1,26	9,01	5,35
	72	mean	83,98	107,27	104,89	105,10	87,49	88,37
		CV	1,48	17,21	2,56	9,08	2,04	11,81
	96	mean	114,44	119,12	118,76	105,41	98,32	67,52
		CV	13,37	13,13	14,67	16,63	7,25	5,64
	144	mean	134,61	167,08	132,42	164,12	133,25	110,74
		CV	28,60	24,11	8,16	29,47	12,62	12,28

Tab. 4c: Chang

DKSFM-supplements	Time (h)		Concentrations (%)					
			0,0125	**0,025**	**0,05**	**0,1**	**0,2**	**0,4**
HPMC	**24**	mean	117,25	121,05	113,00	128,88	130,90	113,14
		CV	5,28	4,17	11,40	4,45	8,62	5,30
	48	mean	126,74	117,13	120,69	141,50	135,87	139,28
		CV	5,69	7,80	20,10	3,56	16,34	6,00
	72	mean	83,38	109,04	100,96	118,73	111,21	93,82
		CV	25,63	5,34	3,60	3,85	2,99	0,47
	96	mean	112,29	145,93	133,10	128,53	142,30	102,44
		CV	6,06	23,62	15,17	6,09	5,03	9,38
	144	mean	92,79	132,46	126,09	122,62	110,32	126,70
		CV	8,78	18,68	11,86	5,93	7,22	13,15
Carbopol	**24**	mean	111,37	99,01	111,02	94,96	34,99	8,97
		CV	16,10	11,74	11,67	8,69	0,85	0,67
	48	mean	133,15	114,78	129,29	82,73	17,73	2,00
		CV	11,37	11,11	7,87	5,63	1,12	0,61
	72	mean	95,68	99,81	81,79	92,53	26,33	0,29
		CV	10,83	13,44	8,19	3,08	0,70	0,03
	96	mean	128,51	142,26	105,00	42,87	4,15	-0,09
		CV	15,84	21,36	9,50	8,84	1,28	0,13
	144	mean	82,46	78,92	59,07	22,68	0,77	-0,19
		CV	7,01	25,38	2,58	2,97	0,23	0,02
SH	**24**	mean	95,14	99,94	102,24	82,82	-0,08	-0,15
		CV	5,30	7,89	5,80	10,81	0,01	0,03
	48	mean	99,57	110,48	87,46	49,43	-0,10	-0,02
		SD	5,41	7,37	1,13	3,77	0,03	0,03
	72	mean	123,12	135,23	139,25	107,64	-0,11	-0,03
		CV	4,32	9,96	7,47	5,20	0,01	0,10
	96	mean	68,05	68,97	75,11	39,51	-0,04	-0,11
		CV	1,43	4,02	12,13	12,24	0,08	0,03
	144	mean	84,88	77,42	78,46	16,07	-0,08	-0,12
		CV	4,68	9,05	17,61	11,24	0,10	0,02
allantoin	**24**	mean	93,64	91,33	87,12	97,39	82,70	49,35
		CV	6,21	8,16	6,29	4,10	2,12	7,30
	48	mean	113,39	108,11	102,39	95,32	97,82	73,83
		CV	4,67	2,90	5,52	12,58	8,22	2,67
	72	mean	94,87	94,59	88,78	100,34	89,63	70,80
		CV	6,39	11,92	2,20	3,74	9,06	6,04
	96	mean	76,07	82,49	53,19	66,28	56,31	37,49
		CV	13,56	20,21	6,64	6,02	7,58	3,81
	144	mean	86,03	89,55	95,83	66,93	50,25	41,56
		CV	1,18	19,20	2,95	12,35	5,87	7,57

Tab. 4d: IOBA-NHC

DKSFM-supplements	Time (h)		Concentrations (%)					
			0,0125	**0,025**	**0,05**	**0,1**	**0,2**	**0,4**
HPMC	**24**	mean	121,75	143,23	151,53	147,85	126,79	107,27
		CV	12,53	12,92	14,88	3,67	10,54	4,58
	48	mean	142,26	146,39	135,81	125,05	131,59	124,81
		CV	20,66	28,54	16,39	20,47	19,49	4,17
	72	mean	125,80	109,62	92,93	101,84	92,24	71,32
		CV	20,37	18,90	5,63	16,89	10,80	3,91
	96	mean	91,34	101,19	112,79	96,80	95,75	66,54
		CV	0,18	12,03	7,84	14,68	9,02	6,02
	144	mean	115,50	118,22	127,40	111,44	78,26	73,45
		CV	3,09	2,67	6,91	13,92	9,67	19,07
Carbopol	**24**	mean	176,11	177,75	178,20	170,79	70,84	24,09
		CV	5,39	19,59	16,58	16,58	8,94	5,09
	48	mean	153,31	175,38	171,40	72,92	22,90	1,72
		CV	7,72	14,86	21,38	3,86	4,90	1,57
	72	mean	97,75	100,99	102,65	64,10	12,05	1,71
		CV	5,27	9,25	7,41	8,37	0,54	0,97
	96	mean	79,28	86,12	84,99	30,60	2,27	0,03
		CV	0,67	14,25	13,78	7,41	1,68	0,23
	144	mean	122,10	120,34	91,49	46,71	1,38	-0,33
		CV	7,59	3,72	10,30	5,74	0,13	0,02
SH	**24**	mean	108,66	93,32	106,20	6,85	-0,03	-0,12
		CV	8,67	1,90	6,67	3,03	0,03	0,04
	48	mean	136,31	152,82	146,13	-0,21	-0,21	-0,27
		SD	7,80	8,98	3,10	0,10	0,09	0,03
	72	mean	95,64	90,27	87,30	10,84	-0,04	-0,13
		CV	7,52	7,78	6,06	3,67	0,06	0,04
	96	mean	87,37	86,23	88,76	-0,07	-0,16	-0,27
		CV	7,47	3,05	5,16	0,07	0,10	0,03
	144	mean	99,56	83,51	95,22	-0,07	-0,17	-0,29
		CV	5,34	9,15	1,28	0,09	0,10	0,04
allantoin	**24**	mean	103,44	112,34	114,63	99,64	97,00	75,33
		CV	2,60	7,11	1,80	7,22	4,02	6,87
	48	mean	71,04	93,10	79,28	61,15	87,27	41,46
		CV	7,10	6,74	8,57	6,47	1,76	0,08
	72	mean	103,44	112,34	114,63	99,64	97,00	75,33
		CV	2,60	7,11	1,80	7,22	4,02	6,87
	96	mean	91,45	90,34	84,24	74,35	50,17	30,32
		CV	11,73	15,18	10,62	9,56	2,78	3,10
	144	mean	117,95	123,19	115,08	57,79	49,54	36,47
		CV	3,35	5,45	10,91	3,11	9,00	2,40

Tab. 5: Calcein AM/EthD-1 assay for HCE-T and IOBA-NHC cells treated with DKSFM-variations for 24 or 72 h. The values are presented as mean and CV of relative cell viability (%) for triplicate samples and reflect the amount of green fluorescence measured.

Tab. 5a: HCE-T

DKSFM-supplements	Time (h)		Concentrations (%) 0,0125	0,025	0,05	0,1	0,2	0,4
HPMC	24	mean	101,09	95,16	104,02	108,52	79,99	100,10
		CV	0,40	1,85	0,49	1,17	30,57	5,38
	72	mean	87,11	94,77	96,51	97,12	90,64	65,71
		CV	1,96	2,61	0,44	2,27	2,12	13,09
Hypromellose®	24	mean	95,83	95,57	87,88	71,20	47,61	2,72
		CV	0,56	4,18	9,80	1,23	15,42	7,93
	72	mean	101,69	102,49	91,47	58,01	27,59	5,33
		CV	2,86	0,50	6,12	20,47	4,95	2,18
Carbopol	24	mean	88,38	94,80	97,90	86,18	61,88	13,29
		CV	16,87	17,89	11,89	9,48	6,14	17,01
	72	mean	104,56	110,35	95,16	84,09	59,71	9,09
		CV	6,46	2,32	0,28	3,65	16,24	5,30
Thilo-Tears®	24	mean	81,49	87,97	49,64	23,21	1,97	1,24
		CV	22,93	15,71	6,07	11,53	4,76	1,07
	72	mean	98,05	111,04	84,55	25,57	4,64	0,14
		CV	11,00	1,17	0,18	3,00	2,12	1,72
SH	24	mean	99,94	101,16	99,17	99,05	96,97	2,17
		CV	1,76	1,90	0,36	1,77	1,86	4,19
	72	mean	93,99	93,67	84,40	72,76	68,88	0,47
		CV	1,79	0,20	0,23	1,30	1,61	1,76
Vislube®	24	mean	95,61	101,81	101,42	85,29	37,48	-
		CV	2,52	0,33	1,76	3,87	11,47	-
	72	mean	81,16	80,26	71,28	66,14	14,40	-
		CV	0,33	0,59	0,55	0,94	8,89	-
allantoin	24	mean	82,80	78,02	84,43	96,05	98,06	69,42
		CV	12,43	12,27	8,44	8,60	11,07	5,27
	72	mean	87,62	87,35	87,64	88,81	91,10	62,55
		CV	0,17	3,33	0,18	1,29	5,04	6,99

Tab. 5b: IOBA-NHC

DKSFM-supplements	Time (h)		Concentrations (%) 0,0125	0,025	0,05	0,1	0,2	0,4
HPMC	**24**	mean	89,21	90,46	84,69	89,58	88,60	87,68
		CV	1,40	1,71	1,03	4,28	0,53	1,30
	72	mean	120,87	109,19	104,13	93,90	100,83	102,38
		CV	2,36	0,68	6,78	2,70	5,74	3,37
Hypromellose®	**24**	mean	86,97	83,03	84,66	91,85	28,57	33,92
		CV	2,97	0,41	0,47	0,18	16,83	0,88
	72	mean	92,13	91,77	91,27	84,17	19,86	36,62
		CV	0,74	0,79	1,72	1,20	4,20	8,70
Carbopol	**24**	mean	98,48	98,15	84,22	101,12	73,42	18,47
		CV	0,13	1,14	0,59	9,27	1,03	54,85
	72	mean	110,31	96,37	130,27	93,85	34,45	15,81
		CV	2,81	2,44	2,29	1,18	14,35	5,42
Thilo-Tears®	**24**	mean	98,99	92,85	92,09	91,88	6,97	2,87
		CV	3,26	0,72	2,04	13,28	25,42	3,87
	72	mean	102,30	93,50	97,65	20,51	1,69	3,51
		CV	10,14	7,34	14,23	1,96	2,37	35,63
SH	**24**	mean	88,12	92,95	79,88	81,38	77,71	38,62
		CV	1,12	1,62	1,76	2,42	1,14	11,35
	72	mean	93,85	92,19	92,86	90,61	95,83	23,86
		CV	0,01	0,42	2,86	4,04	11,02	22,51
Vislube®	**24**	mean	87,35	86,32	90,70	95,24	91,59	-
		CV	2,50	3,73	2,32	1,39	0,14	-
	72	mean	92,86	86,93	91,42	87,28	75,41	-
		CV	0,78	4,35	1,21	0,82	0,93	-
allantoin	**24**	mean	98,25	100,15	95,57	96,04	94,80	93,40
		CV	3,69	1,64	4,01	3,13	3,05	1,33
	72	mean	107,27	110,65	101,31	102,78	93,53	100,30
		CV	2,61	2,89	4,01	3,29	2,73	1,72

Tab. 6: ATP and Calcein AM/EthD-1 assays for IOBA-NHC cells treated with DKSFM-variations and artificial tears for 24 h. The values are presented as mean and CV of relative cell growth (%) for triplicate samples.

DKSFM-Supplements	Assays		Concentrations (%)					
			0,0125	0,025	0,05	0,1	0,2	0,4
HPMC	Calcein AM/EthD-1	mean	89,21	90,46	84,69	89,58	88,60	87,68
		CV	1,40	1,71	1,03	4,28	0,53	1,30
	ATP	mean	121,75	143,23	151,53	147,85	126,79	107,27
		CV	10,96	11,65	13,03	3,67	9,38	4,57
Hypromellose®	Calcein AM/EthD-1	mean	86,97	83,03	84,66	91,85	28,57	33,92
		CV	2,97	0,41	0,47	0,18	16,83	0,88
	ATP	mean	104,29	68,58	119,82	94,56	37,61	0,08
		CV	4,39	7,75	4,93	8,53	3,65	0,04
Carbopol	Calcein AM/EthD-1	mean	98,48	98,15	84,22	101,12	73,42	18,47
		CV	0,13	1,14	0,59	9,27	1,03	54,85
	ATP	mean	176,11	177,75	178,20	170,79	70,84	24,09
		CV	4,96	18,52	16,55	14,31	8,92	4,88
Thilo-Tears®	Calcein AM/EthD-1	mean	98,99	92,85	92,09	91,88	6,97	2,87
		CV	3,26	0,72	2,04	13,28	25,42	3,87
	ATP	mean	161,03	179,80	153,59	87,17	3,02	-0,17
		CV	5,23	13,59	13,93	16,58	1,47	0,05
SH	Calcein AM/EthD-1	mean	88,12	92,95	79,88	81,38	77,71	38,62
		CV	1,12	1,62	1,76	2,42	1,14	11,35
	ATP	mean	108,66	93,32	106,20	6,85	-0,03	-0,12
		CV	7,83	1,88	6,65	2,63	0,03	0,03
Vislube®	Calcein AM/EthD-1	mean	87,35	86,32	90,70	95,24	91,59	-
		CV	2,50	3,73	2,32	1,39	0,14	-
	ATP	mean	91,53	92,83	107,01	108,21	24,28	-
		CV	2,81	5,78	11,53	1,12	1,18	-

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