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Impact of Photodynamic Therapy (PDT) on Human Keratocytes and Corneal Endothelial Cells in Vitro

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Für meine geliebten Eltern

To my beloved parents

献给我深爱的父母

Declaration

I hereby declare that this thesis is my own original work and effort. All experiments, except for those specified, were exclusively performed by me. Except for the publications written by myself listed in the publication list, the data presented here have not been submitted anywhere else for any award. Where other sources of information and help have been used, they have been indicated and acknowledged.

Homburg/Saar, 20.05.2013

Jiong Wang

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LIST OF ABBREVIATIONS

5-aminolevulinic acid	ALA
Alpha-smooth muscle actin	α-SMA
Antibiotic resistance monitoring in ocular microrganisms	ARMOR
Basic fibroblast growth factor	FGFb
Benzoporphyrin derivative	BpD
Bovine serum albumin	BSA
Chlorin e6	Ce6
Cluster of differentiation 34	CD34
Coagulase-negative staphylococcus	CNS
Corneal collagen crosslinking	CLX
Drug-resistant streptococcus pneumoniae	DRSP
Dulbecco's modified eagle medium: nutrient mixture F-12	DMEM/F12
Ethylenediaminetetra-acetic acid	EDTA
Fetal bovine serum	FBS
First excited singlet state	S1
Hematoporphyrin derivative	HpD
Hepatocyte growth factor	HGF
Herpes simplex virus	HSV
Human corneal endothelial cells	HCECs
Including hydrogen peroxide	H_2O_2
Infrared rays	IR

Keratinocyte growth factor	KGF
Methicillin-resistant staphylococcus aureus	MRSA
Multidrug-resistant pseudomonas aeruginosa	MDRPA
One-way analysis of variance	ANOVA
Penicillin/streptomycin	P/S
Phosphate-buffered saline	PBS
Photodynamic therapy	PDT
Photosensitizer	PS
Photosensitizers	PSs
Propidium iodide	PI
Pseudomonas aeruginosa	PA
Reactive oxygen species	ROS
Reactive singlet oxygen	$^1O_2$
Second excited singlet state	S2
Simian virus 40	SV40
Singlet state	S0
Standard deviation	SD
Staphylococcus aureus	SA
Staphylococcus epidermidis	SE
Superoxide anion	O_2^-
Transforming growth factor β1	TGFβ1
Toll-like receptor	TLR
Ultraviolet	UV
Vascular endothelial growth factor	VEGF

SUMMARY

Impact of Photodynamic Therapy (PDT) on human keratocytes and corneal endothelial cells in vitro

Purposes: With increasing resistance of microorganisms to antibiotics, PDT may be a potential alternative in case of therapy resistant infectious keratitis. PDT is based on a photosensitizer activated by light of appropriate wavelength, which leads to generation of singlet oxygen and free radicals, responsible for the cytotoxic effect on microorganisms. Thus, it is important to determine the influence of PDT on human keratocytes and corneal endothelial cells.

The *purpose* of this study was:

- To evaluate the impact of PDT using the photosensitizer Ce6 on viability, apoptosis, proliferation and activation of human keratocytes, in vitro.
- To investigate the impact of PDT using the photosensitizer Ce6 on the secretion of KGF, FGFb, VEGF, HGF and TGFβ1 of human keratocytes, in vitro.
- To determine the impact of PDT using the photosensitizer Ce6 on viability, apoptosis and proliferation of human corneal endothelial cells (HCECs), in vitro.

Methods: Primary human keratocytes were isolated by digestion in collagenase (1.0 mg/ml) from human corneal buttons and cultured in DMEM/Ham's F12 medium supplemented with 10% fetal calf serum (FCS). An immortalized human corneal endothelial cell line was cultured in DMEM/Ham's F12 medium supplemented with

5% FCS. Cell cultures underwent illumination using red (670 nm) light for 13 minutes with a dose of 24 J/cm^2 following exposure to 0 nM-64 μM concentrations of Ce6 in the culture medium.

Twenty-four hours after PDT, human keratocyte and HCECs viability was evaluated by the Alamar blue assay, apoptosis and total DNA content of the cells using the APO- DIRECT ^{TM} Kit and cell proliferation by the BrdU Cell Proliferation Assay Kit. CD34 and alpha smooth muscle actin (α-SMA) expression of human keratocytes was analysed using flow cytometry (FACS) after illumination following exposure to 50, 150 and 250 nM concentrations of Ce6 in the culture medium. In addition, five and twenty-four hours after illumination following exposure to 100 nM concentration of Ce6, the secretion of KGF, FGFb, VEGF, HGF and TGFβ1 of human keratocytes was measured by enzyme-linked immunoabsorbent assay (ELISA).

Results: Using Ce6 or illumination only, we did not detect significant changes of human keratocyte and HCECs viability, apoptosis and proliferation. Following PDT, viability and total DNA content of human keratocytes decreased significantly above 100 nM and 150 nM Ce6 concentration ($P<0.01$; $P<0.01$), respectively. The percentage of apoptotic keratocytes increased significantly from 250 nM Ce6 concentration ($P<0.01$) and proliferation of keratocytes decreased significantly ($P<0.05$) above 100 nM concentration of Ce6 after PDT.

Using Ce6 or illumination only, CD34 and α-SMA expression of human keratocytes did not change significantly. Twenty-four hours after PDT, the percentage of α-SMA positive human keratocytes decreased significantly at 250 nM concentration of Ce6 ($P=0.02$).

Compared to untreated controls, FGFb secretion of human keratocytes increased

(P<0.01) and HGF expression decreased (P<0.01) significantly 5 hours after PDT, whereas KGF, VEGF, and TGFβ1 secretion remained unchanged. Twenty-four hours following PDT, KGF secretion decreased (P<0.01) significantly, while FGFb, HGF, VEGF and TGFβ1 concentrations did not differ markedly from untreated controls.

In HCECs, viability and total DNA content decreased significantly above 150 nM Ce6 concentration (P<0.01; P<0.05). The percentage of apoptotic HCECs increased significantly from 250 nM Ce6 concentration (P<0.01) and proliferation of endothelial cells decreased significantly (P<0.05) above 100 nM concentration of Ce6 after PDT.

Conclusions:

- PDT using Ce6 decreases viability and proliferation, suppresses myofibroblastic transformation and triggers apoptosis of human keratocytes, in vitro.
- PDT triggers FGFb and inhibits HGF secretion of human keratocytes 5 hours and inhibits KGF secretion 24 hours following treatment.
- PDT using Ce6 decreases viability and proliferation, and also triggers apoptosis of HCECs, in vitro.

ZUSAMMENFASSUNG

Einfluss der photodynamischen Therapie (PDT) auf humane Keratozyten und korneale Endothelzellen in vitro

Ziele: Durch die zunehmende Resistenzbildung von Bakterien gegenüber Antibiotika kann die Behandlung mit Photodynamischer Therapie (PDT) ein Verfahren zur Therapie einer infektiösen Keratitis darstellen. Die Wirkung der PDT basiert auf der Kombination eines von einer bestimmten Wellenlänge angeregten Photosensibilisators und des dadurch resultierenden Zelltodes durch freie Sauerstoffradikale. Aufgrund dieser Wirkungsweise ist es wichtig, den Einfluss der PDT auf humane Keratozyten und korneale Endothelzellen zu evaluieren.

Die Ziele dieser Studie waren:

- Den Einfluss der PDT unter Verwendung des Photosensibilisators Ce6 auf die Viabilität, Apoptose, Proliferation und Aktivierung kultivierter humaner Keratozyten auszuwerten.
- Die Wirkung der PDT unter Verwendung des Photosensibilisators Ce6 auf die Sekretion von KGF, FGFb, VEGF, HGF und TGFβ1 zu untersuchen.
- Den Einfluss der PDT unter Verwendung des Photosensibilisators auf die Viabilität, Apoptose und Proliferation humaner kornealer Endothelzellen (HCECs) in der Zellkultur auszuwerten.

Methoden: Humane primäre Keratozyten wurden durch enzymatische Behandlung mit Collagenase A (1,0 mg/ml) aus humanen Korneoskleralscheiben isoliert und in DMEM/Ham's F12 Kulturmedium, versetzt mit 10% fetalem Kälberserum kultiviert. Die immortalisierte humane korneale Endothelzelllinie wurde in DMEM/Ham's F12 Kulturmedium, versetzt mit 5% fetalem Kälberserum kultiviert. Die Zellkulturen wurden mit Ce6 in einer Konzentration von 0 nM-64 µM für 30 Minuten inkubiert und anschließend mit einer Wellenlänge von 670 nm und einer Energiedosis von 24 J/cm^2 für 13 Minuten bestrahlt.

24 Stunden nach der Behandlung erfolgte die Messung der Viabilität der humanen Keratozyten und der kornealen Endothelzellen mit dem AlamarBlue assay, die Proliferation wurde mit einem Zell-Proliferations-ELISA bestimmt, und die Apoptose durchflusszytometrisch mit dem APO-DIRECT™ Kit ermittelt.

Die Erfassung der Expression von CD34 und alpha smooth muscle actin (α-SMA) erfolgte durchflusszytometrisch (FACS) 24 Stunden nachdem die humanen Keratozyten mit Ce6 in den Konzentrationen von 0, 50, 150 und 250 nM für 30 Minuten inkubiert und anschließend mit einer Wellenlänge von 670 nm für 13 Minuten bestrahlt wurden.

Fünf und 24 Stunden nach der Behandlung (mit einer Konzentration von 100 nM Ce6) wurde die Sekretion von KGF, FGFb, VEGF, HGF und TGFβ1 mit einem enzym-linked immunoabsorbent assay (ELISA) photometrisch analysiert.

Ergebnisse: Die ausschließliche Anwendung von Ce6 oder Bestrahlung führte nicht zu einer signifikanten Veränderung der Viabilität, Apoptose oder Proliferation bei den humanen Keratozyten oder den kornealen Endothelzellen. Die Viabilität und der DNA-Gehalt der humanen Keratozyten sanken mit einer Ce6 Konzentration von 100

nM und 150 nM signifikant (P<0,01; P<0,01). Die Apoptoserate der Keratozyten stieg ab einer Ce6 Konzentration von 250 nM Ce6 signifikant an (P<0,01). Darüberhinaus führte die PDT ab einer Ce6 Konzentration von 100 nM zu einer signifikant verminderten Proliferationsrate der Keratozyten (P<0,05).

Unter Verwendung von entweder nur Ce6 oder nur Bestrahlung ergaben sich keine Veränderungen der Expression von CD34 und α-SMA der Keratozyten. Vierundzwanzig Stunden nach PDT mit 50-250 nM Ce6 blieb die CD34 Expression unverändert, während der Prozentsatz von α-SMA positiven Keratozyten bei 250 nM Ce6 signifikant sank (P=0,01).

Fünf Stunden nach der Behandlung blieb die Sekretion von KGF, VEGF und TGFβ1 unverändert, während die FGFb Sekretion der Keratozyten anstieg (P<0,01), und die HGF Sekretion sank (P<0,01). Nach 24 Stunden sank die KGF Sekretion signifikant (P<0,01), während die Konzentrationen von FGFb, HGF, VEGF und TGFβ1 im Vergleich zu den Kontrollen unverändert blieben.

Bei den kornealen Endothelzellen sanken die Viabilität und der DNA-Gehalt ab einer Ce6 Konzentration von 150 nM signifikant (P<0,01). Die Apoptoserate der kornealen Endothelzellen stieg ab einer Ce6 Konzentration von 250 nM Ce6 signifikant an (P<0,01). Darüberhinaus führte die PDT ab einer Ce6 Konzentration von 100 nM zu einer signifikant verminderten Proliferationsrate der Endothelzellen (P<0,05).

Schlussfolgerung:

- PDT unter Verwendung von Ce6 senkt die Viabilität und Proliferationsrate, hemmt die myofibroblastische Transformation und steigert die Apoptoserate von humanen Keratozyten in der Zellkultur.
- PDT steigert die Sekretion von FGFb und vermindert die Sekretion von HGF von

Keratozyten 5 Stunden nach der Behandlung, 24 Stunden nach PDT zeigt sich eine erniedrigte KGF Sekretion.

- PDT unter Verwendung von Ce6 senkt die Viabilität und Proliferationsrate von kornealen Endothelzellen, und steigert deren Apoptoserate in der Zellkultur.

1 INTRODUCTION

The eye, called the window of the soul is a sensory organ for the perception of light stimuli. A healthy, clear cornea is essential for good vision of the eye as it is the anterior front part of the eye in front of lens, vitreous body and retina. The cornea contributes to approximately three-quarters of the total optical power of the eye. [1] Corneal opacities due to infection, injury or any other ocular surface diseases interfere with vision.

Infectious keratitis may be caused by bacteria, fungi, viruses and protozoa. If the appropriate diagnosis and antimicrobial treatment are delayed, only about 50% of eyes gain good visual recovery. [2] Furthermore, treatment failure may even happen despite the use of broad spectrum antibiotics in clinical routine. Therefore, researchers try to explore potential alternative therapies for infectious keratitis, including especially those with resistance to antimicrobial treatment.

In this work, we describe the challenges in diagnosis and treatment of infectious keratitis. We also define PDT, which may be considered as a potential alternative or additional antimicrobial treatment modality in infectious keratitis.

1.1 Corneal anatomy

1.1.1 General aspects

The normal cornea is mostly horizontally elliptical with an average diameter of 11.5 mm vertically and 12.0 mm horizontally. The average thickness of the central cornea is 0.54 mm, and it becomes thicker towards the periphery. [1]

The cornea is free of blood vessels and is nourished from the aqueous humor of the anterior chamber and through the tear fluid. The cornea is innervated through a subepithelial and a deeper stromal plexus, which are richly supplied by numerous sensory nerve endings of the first division of the trigeminal nerve.

1.1.2 Corneal layers

The human cornea consists of five layers, from anterior to posterior the epithelium, Bowman's layer, the stroma, Descemet's membrane and the endothelium. Each layer plays an important and distinct role in corneal physiology and pathophysiology.

1) Epithelium

The corneal epithelium is the outermost layer of the cornea. It is on average 0.04-0.06 mm thick and is composed of multiple epithelial cell layers. There are three different types of cells: the basal, wing and squamous cells. The basal cells are adhered to the underlying basement membrane. Above them, there are two or three layers of wing cells and two layers of superficial squamous cells, adjacent to the tear film. The superficial cells with many microplicae and microvilli are involved in the stabilization of the tear film and the absorption of nutrients. In

addition, highly resistant tight junctions formed between neighboring epithelial cells provide a protective barrier, which is essential for good corneal optical properties during continuous renewal of the corneal epithelium. [3,4]

Limbal stem cells are indispensable for the maintenance of a healthy corneal surface. These are principally located at the basal layer of the palisades of Vogt at the cornea conjunctival junction. [5-7] The deficiency of limbal stem cells may result in conjunctival epithelial ingrowth, neovascularization, chronic inflammation or recurrent epithelial erosions and defects. [8,9]

2) Bowman's layer

The corneal Bowman's layer is an acellular superficial layer of the stroma consisting of collagen fibers. The Bowman's layer is a visible indicator of ongoing stromal-epithelial interactions in the cornea. However, its critical function in corneal physiology is still unclear. [10]

3) Stroma

The stroma is the transparent middle layer of the cornea, which is about 0.40-0.50 mm thick. It accounts for about 90% of the total corneal thickness. The corneal stroma is also called substantia propria. It comprises arranged collagen fibers with sparsely distributed interconnecting keratocytes. The maintenance of lattice arrangement and spacing of collagen fibrils is critical for the transparency of the cornea. [11,12]

Under normal circumstances, stromal keratocytes are rarely dividing cells. However, keratocytes can proliferate and migrate in case of tissue damage and transform into activated fibroblasts or/and myofibroblasts. They also produce

collagen and proteoglycans for repair when an injury occurs in the stroma. [13]

4) Descemet's membrane

The Descemet's membrane consists of a fine latticework of collagen fibrils, which are distinct from the collagen lamellae of corneal stroma. It serves as a protective barrier against infection and injuries. Descemet's membrane contains 3 zones: an anterior perinatal zone which is deposited in utero; a middle "normal" adult zone; a posterior abnormal zone, which serves as a modified basement membrane for the endothelium. [14]

5) Endothelium

The corneal endothelium is approximately 0.005 mm thick. It is a monolayer of cells at the posterior surface of the cornea. Endothelium is essential in keeping the cornea transparent, as it is responsible for a dynamic balance between a “leaky” barrier for fluid moving into the stroma and a fluid pump to actively move fluid from the stroma back into the anterior chamber of the eye. [15-17]

Corneal endothelial cells have a limited regenerative potential in vivo. The cell density in human adult is approximately 2500 cells/mm². This number steadily decreases with advancing age, [18] diseases [19] and intraocular surgery. [20]

Maintenance of corneal transparency can be lost when endothelial cell density is below a critical level.

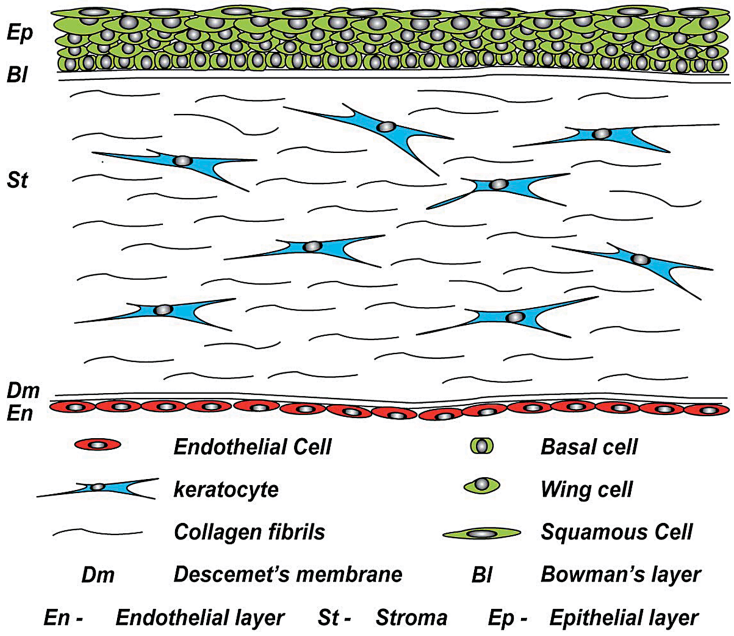

Figure 1. The human cornea in cross-section. [4] At the outer surface of the cornea, there is an epithelial layer adhered to a basement membrane above Bowman's layer. The stromal layer is sparsely populated with keratocytes. The single sheet of endothelial cells sits on Descemet's membrane.

1.2 Infectious keratitis

1.2.1 Definition of infectious keratitis

Infectious keratitis is an inflammatory condition of the cornea, characterized by inflammation in any layer of the cornea caused by an infection involving bacteria, viruses, fungi or protozoa. [1] Infectious keratitis is an emergency condition with patients often suffering from significant pain, distress, and acute visual loss.

1.2.2 Epidemiology

The incidence of infectious keratitis per 100 000 person-years in the *developed world* was 3.6 in the West of Scotland, [21] 6.3 in Hong Kong [22] and 27.6 in Northern California. [23] However, it has been increasing due to higher rates of contact lens use. In Northern California, the incidence was 14.0 in non-contact lens wearers, whereas 130.4 in contact lens wearers. [23] The incidence of acanthamoeba keratitis is also in closed association with contact lens use and it was 14.9 per 100 000 soft contact lens wearers. [21] In developed countries, herpetic eye disease is the most common cause of infectious keratitis, about 60% of infectious keratitis in developed countries may be the result of herpes simplex virus. [1]

The incidence of infectious keratitis per 100 000 person-years in the *developing world* is considerably higher, e.g. it was 113 in India [24] and 799 in Nepal. [25] Among severe infectious corneal ulcers, fungal keratitis is the most common infectious keratitis in the developing countries like China, India and Nepal. [25-27] One retrospective analysis from south India, aiming to determine the prevalence of microbial pathogens causing keratitis, has shown that 32.7% of infectious keratitis was due to bacterial, 34.4% due

to fungal aetiology. [27] Meanwhile, one epidemiologic study from north China between 1999 and 2004 on infectious keratitis has reported of 61.9% fungal, 12.2% bacterial and 1.4% acanthamoeba keratitis and no pathogens could be isolated in 24.5% of the cases. [26]

1.2.3 Etiology

The risk factors for infectious keratitis include overnight or extended wear of contact lenses, orthokeratology lenses, ocular trauma, ocular surface disease (including atopic or vernal keratoconjunctivitis and blepharitis), previous ocular surgery, previous keratitis (herpes simplex keratitis), systemic diseases (diabetes mellitus, rheumatoid arthritis) and the use of topical corticosteroids. [22,28-33] However, in about 7.9% of individuals with infectious keratitis no identifiable risk factors could be verified. [29] Among infectious keratitis cases, the contact lens related and traumatic keratitis are more common in younger patients, whereas the keratitis associated with prior ocular surgery or ocular surface disease is more common in older persons. [34]

Common causative organisms associated with bacterial keratitis are from the gram positive group *Staphylococcus aureus* (*SA*) and *coagulase-negative Staphylococcus* (*CNS*) and from the gram-negative group *Pseudomonas aeruginosa* (*PA*). Some studies reported that *PA* is the most common organism in positive cultures of corneal scrapings: 20.1% in China, [35] 25.7% in South Florida [36] and 17% in Brisbane, Australia. [34] However, this prevalence of causative organisms is not in accordance with other geographical regions, which found *CNS* to be the most common organism 30% isolated on corneal scrapings in six centers in the United Kingdom between 2003 and 2006 [37] and 21.8% of 201 cases in France between 1998 and 1999. [38] *SA* (26.7%) was the most common isolated pathogen from bacterial keratitis in South India

between 2002 and 2007. [39] Green et al. [34] reported that *PA* (55%) is the most common pathogen isolated from contact lens related cases, *SA* (29%) is more common in ocular surgery related cases, and negative culture isolates (59%) are more common in traumatic keratitis.

Among fungal keratitis, *filamentous fungi* are the most common causative microorganism associated with trauma caused through plants in the developing countries, [26,27,40,41] and contact lens wear in the United States, [42,43] whereas *yeast* is more frequently the causative pathogen in patients with ocular surface diseases.

1.2.4 Clinical features and diagnosis

Early symptoms of infectious keratitis include redness, tearing, pain, sensitivity to light, purulent discharge, a white corneal infiltrate and decreased vision. However, certain signs may also be unique or specific presentations for one special kind of infectious keratitis.

In *fungal keratitis* serrated margins, raised slough, dry texture, satellite lesions and coloration other than yellow were observed more frequently. [44] The probability of fungal infection was 63% if one clinical feature was present, increasing to 83% if all three features were present.

In cases of *acanthamoeba keratitis* the clinical features are photophobia, disproportionate pain of the cornea, a radial pattern of perineural infiltrates, ring infiltrates, a central epithelial loss with stromal thinning and occasionally progression to corneal melt. [45] Acanthamoeba keratitis, with the common disciform epithelial and stromal infiltrate features, was commonly initially diagnosed as herpes simplex virus (HSV) or even fungal keratitis. [45]

A provisional diagnosis for infectious keratitis can be made using the history and the

clinical features, although a definitive diagnosis requires conventional methods of culture and histology, advanced noninvasive techniques [46-48] and molecular techniques for diagnosis of viral and fungal keratitis. [49,50] Corneal scraping provides samples for an etiological diagnosis, moves necrotic tissue and enhances antibiotic penetration.

In the absence of a clear diagnosis, diagnostic treatment ex juvantibus can even be considered. If the infectious keratitis is running with an atypical presentation and course, or it is found unresponsive to the initial medical treatment, the possibility of a co-infection should be considered. [51]

1.2.5 Treatment

Antimicrobial agents should be started as soon as diagnostic test were performed. The initial treatment should generally use broad spectrum antibiotics covering most common pathogens. More selective agents are applied if the results of the diagnostic tests are available. Topical steroids, if applied, should always be used with caution as they may promote replication of some types of organisms, specifically herpes simplex virus and fungi.

Besides, surgical treatment is also of great importance for some conditions of infectious keratitis, including removal of infected epithelium to facilitate re-epithelialization and eye drop absorption, transplantation of amniotic membrane or even keratoplasty. [1]

1.2.6 Antibiotic resistance and management

The most common factors contributing to increasing trend of antimicrobial drug resistance among ocular organisms are the overuse of drugs for systemic infection and the widespread and sometimes inappropriate use of topical antibiotics for eye disease.

[52-55] Studies have reported on the increasing resistance of gram positive organisms, [53] *pseudomonas* [56] and *atypical mycobacteria.* [57] Besides the increased resistance during the past decade, the incidence of gram positive organisms, specifically *SA*, has been increasing significantly. [36,58,59] One example for resistant microorganisms is the *methicillin-resistant Staphylococcus aureus (MRSA)*, which is known for its resistance to all available penicillins and other β-lactam antibiotics.

In the 2009 Antibiotic Resistance Monitoring in Ocular MicRorganisms (ARMOR) surveillance study, [60] the common ocular isolates of *SA* and *CNS* were collected from 34 institutions across the United States and analysed by testing the antibacterial susceptibility profile of these microrganisms. The results showed that 39% of *SA* and 52.8% *CNS* isolates were resistant to methicillin, and 46.5% of *SA* and 58.3% of *CNS* isolates were nonsusceptible to 2 or more antibacterial drug classes. Almost all of the *MRSA* isolates were resistant to azithromycin and nearly 80% were resistant to ciprofloxacin. With the growing evidence of resistance to current antibiotics, ophthalmologist try to select a best initial treatment for infectious eye disease with the development of new antibiotics, or effective therapeutic options to treat infectious keratitis. Fortunately, all the through ARMOR study identified *SA* and *CNS* isolates were susceptible to vancomycin.

Furthermore, the use of topical fourth-generation fluoroquinolones and photodynamic therapy (riboflavin/ultraviolet crosslinking) are considered as potential alternatives in the management of infectious keratitis. [61,62] Notably, the antimicrobial efficacy of riboflavin/ultraviolet-A crosslinking for *SA*, *MRSA*, *PA* and fungal isolates was determined. [63,64]

1.3 Photodynamic Therapy

1.3.1 Definition of PDT

Photodynamic therapy (PDT) is a promising treatment for cancer and other diseases. PDT involves the application of a photosensitizer (PS) compound followed by exposure to light of appropriate wavelength. It results in production of reactive oxygen species (ROS) leading to cell death and/or tissue damage.

The first observation of tissue photochemical sensitization by the interaction between light and a chemical drug was reported by Raab [65] in 1900. He discovered a lethal effect on Infusoria, a species of paramecium, dependent on a combination of acridine red and light. Furthermore, Tapperner and Jesionek [66] used a combination of topically applied fluorescent substance of eosin and white light to treat skin tumors, which was considered as the first medical application of PDT. Over the past century, PDT was widely used in clinical treatment. The combination of cell or tissue selective PS uptake and local light exposure provided a potential effective approach to cancer [67] and infectious disease [68] treatment. It has efficient cytotoxicity, but limited damage to the surrounding normal cells or tissues.

1.3.2 Photochemistry of PDT

The photochemical reaction is represented at the Jablonski diagram. [69] Through illumination with light of appropriate wavelength, the PS in a singlet state (S0) is excited from ground state to the first excited singlet state (S1) and the second excited singlet state (S2).

Following excitation, the molecule can relax back to the ground state by emitting

fluorescence or through nonradioactive relaxation quenching processes, but can also be converted to the excited triplet state (T1) with a longer life-time. The T1 can return to the ground state by emitting phosphorescence and can also undergo type I (electron transfer) and/or type II (energy transfer) reactions to produce highly reactive oxygen species (ROS). [67,70]

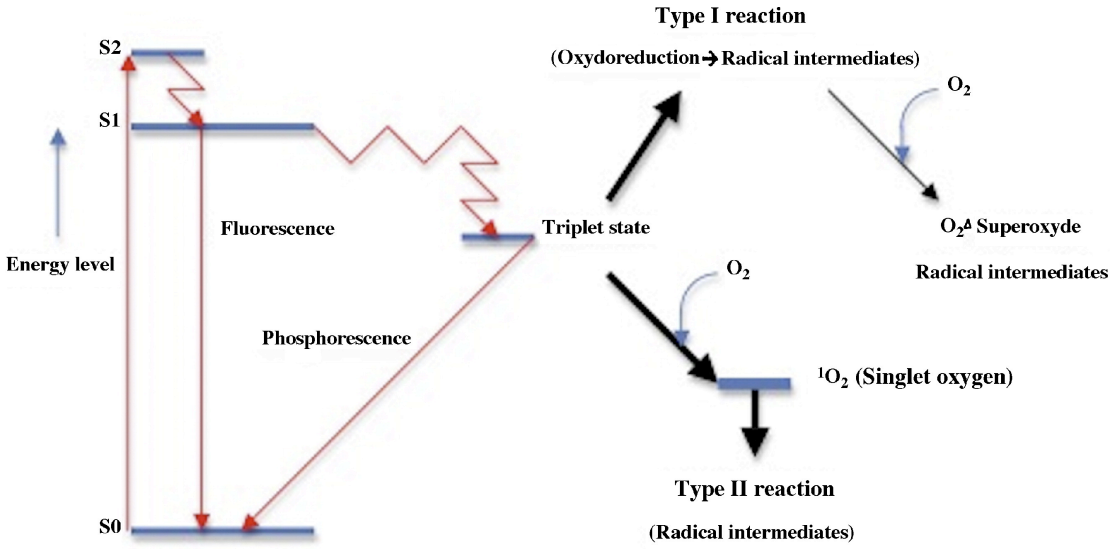

Figure 2. Jablonski diagram representing the excitation and relaxation of a photosensitizer (PS), with type I and type II photoreactions. S0 = ground singlet state; S1 = first excited singlet state; S2 = second excited singlet state. [69]

Generally, in the type I reaction free oxygen radicals formed by the electron-transfer reaction between the excited triplet state and a substrate molecule produce ROS including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). In the type II reaction, the direct energy-transfer of T1 leads to the formation of highly reactive singlet oxygen ($^1O_2$). Singlet oxygen is an electronically excited state of molecular oxygen and has a short life-time in biologic systems and a short radius of action [71]. Thus, the reaction of reactive singlet oxygen can lead to a localized response without

damaging distant cells or tissues. It is not only generally accepted that Type II reactions play a dominant effect in application of PDT in cancer treatments, [72-75] but also seems to be the major pathway in reduction of organism damage in antimicrobial PDT. [76-78]

1.3.3 Light sources

Visible light has a wavelength spectrum within the range of approximately 400-700 nm between the invisible ultraviolet (UV) and the invisible infrared rays (IR). [79]

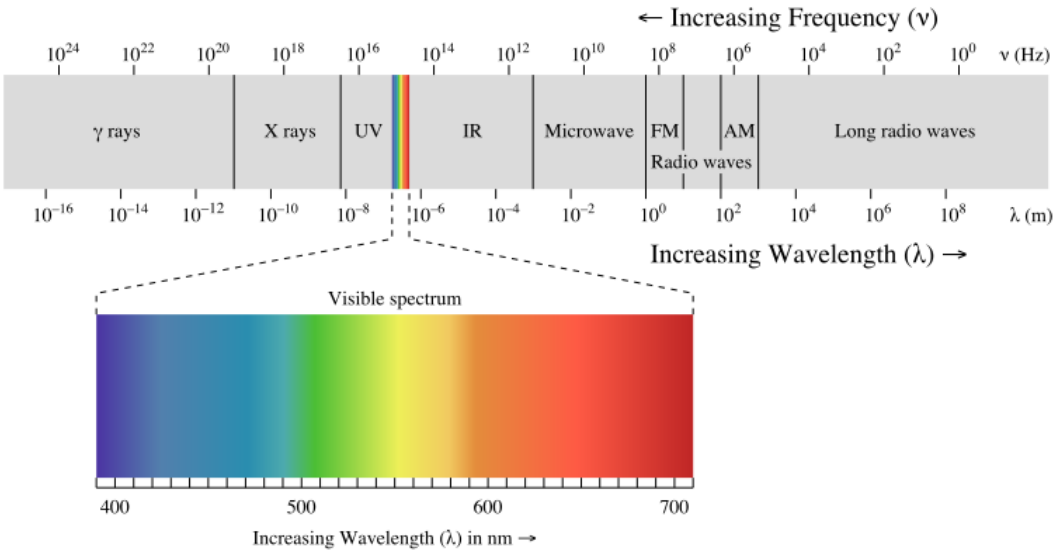

Figure 3. http://en.wikipedia.org/wiki/File:EM_spectrum.svg

The use of light as a therapeutic agent originates from the ancient Egypt, India and China. It was used to treat skin diseases such as psoriasis, vitiligo and also in cancer and psychosis. [80]

PDT requires a source of light to activate the PS. The activating light is most often generated by lasers as they possess a high power density, a monochromatic character (light of one wavelength) and high local and temporal coherence. [81,82] The energy

absorbed by the PS in a volume of tissue, can be calculated exactly if the fluence rate of light, drug concentration and the absorption coefficient are defined at all times in the concrete tissue volume. [83] During PDT, the depth of light penetration into the tissue varies with the optical properties of the tissue and the wavelength of light, which may be a critical factors contributing to efficacy of PDT. [84,85]

1.3.4 Photosensitizers (PSs)

The majority of PSs possess a porphyrin ring structure, which is similar to that of chlorophyll or heme in hemoglobin. The structure of PSs plays a key role in their success as a PDT agent. The PSs absorb light of a specific wavelength, which results in the transfer and translation of light energy into chemical reaction in the presence of molecular oxygen and then in production of singlet oxygen or superoxide. These molecules induce cell damage through direct or indirect cytotoxicity. [86] Therefore, the PS is considered to be a critical component in PDT procedures.

Since the first observation of tissue photochemical sensitization reported by Raab [65] in 1900, many new PSs were synthesized and analysed over the past century.

In clinical application, PSs are divided into three families such as porphyrin, chlorophyll and dye **(Table 1)**. Generally, the porphyrins, developed in the 1970s and early 1980s, are called first generation PSs. Porphyrin derivatives were developed since the late 1980s and are called second generation PSs. Third generation PSs are available drugs modificed with biologic conjugates to increase the affinity of the PS for the tissue. [87,88] The fact that different generations of PSs exist does not mean that newer generation drugs in clinical application are better than older drugs. [86]

Table 1. Photosensitizer families

1.	**Porphyrin platform**
	Hematoporphrin derivative (HpD)
	Benzoporphrin derivative (BpD)
	5-aminolevulinic acid (ALA)
	Texaphrins
2.	**Chlorophyll platform**
	Chlorins
	Punpurins
	Bacteriochlorins
3.	**Dyes**
	Phtalocyanine
	Napthalocyanine

Administration of Hematoporphyrin derivative (HpD) followed by local exposure to red light performed by Dougherty et al. [89] resulted in a partial or complete tumour necrosis in 111 of 113 malignant tumors. To date, HpD is one of the successful PSs in the clinical practice and it is an accepted PDT drug, which can be used for early (superficial) bladder cancer in several countries. In addition, porphyrin has a weak absorption maximum at low wavelength (630 nm) as it results in poor tissue penetration of light.

Chlorin e6 (Ce6) is a natural compound and can be produced by Live chlorella. It is a promising second generation photosensitizer with its appropriate photophysical properties. In addition, Ce6 has a long lifetime in its photoexcited triplet state and a

high absorption in the red range of the visible spectrum (longer than 630 nm). PDT using the photosensitizer Ce6 is currently applied an alternative therapeutic option for a variety of malignant tumors both in vitro and in vivo. [90-93]

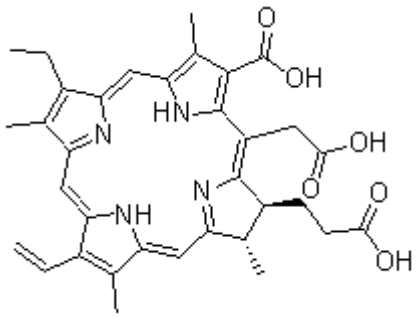

Figure 4. Chlorin e6: 2-[(7S, 8S)-3-Carboxy-7-(2-carboxyethyl)-13-ethenyl-18-ethyl-7, 8-dihydro-2, 8, 12, 17-tetramethyl-21H, 23H-porphin-5-yl] acetic acid. (http://www.medkoo.com/bio-reagents/ChlorinE6.htm)

1.4 PDT in infectious keratitis

The widespread use of antibiotics leads to the increasing resistance of microorganisms to antibiotics. Therefore, the development of potent and safe antimicrobial agents in order to prevent or treat infectious diseases is necessary and led to the use of bacteriophages, [94] naturally occurring or synthetic antimicrobial peptides [95] and antibacterial PDT. [68,96,97]

During 1960's, irreversible photosensitization of entero and even other viruses was described by Wallis and Melnick. [98-101] In 1972, Moore et al. [102] first demonstrated that herpetic keratitis was rapidly resolved following PDT using the PS proflavine in a rabbit model. In contrast, a clinical trial has shown that there was no significant difference in the number of healed patients, the mean healing time, or the number of recurrences of the herpetic corneal ulcers treated by PDT. [103]

In vitro studies demonstrated that *Acanthamoeba* isolates are most sensitive for radiation exposure combined with one potential drug, such as Hypocrellins B, [104] Methylene blue, [105] Tetracationic Zn(II)-phthalocyanine (RLP068) [106] and Riboflavin. [107] The evaluation of clinical cases also revealed that the adjunctive use of riboflavin/UVA crosslinking seems to be a potential alternative for Acanthamoeba keratitis. [108]

The anti-fungal properties of porphyrins have been investigated extensively since the early 1980s. [109] Although PDT using phenothiazinium, porphyrin or riboflavin is effective against fungal isolates from corneal scrapings such as *Candida albicans*, *Fusarium species*, and *Aspergillus fumigatus*, in vivo animal studies and human trials are limited. [110,111] Li et al. [112] recently presented eight cases with fungal keratitis.

None of them required corneal transplantation, following riboflavin/UVA crosslinking: visual acuity improved in six cases, remained unchanged and deteriorated in one case.

PDT is capable of killing bacteria both in vitro and in vivo conditions. [63,113-116] Therefore, it may be also an alternative in case of therapy resistant bacterial keratitis. Martins et al. [63] conducted an in vitro study to demonstrate the antimicrobial properties of riboflavin/UVA crosslinking against common pathogens. They found this treatment to be effective against *SA*, *Staphylococcus epidermidis (SE)*, *MRSA*, *PA*, *multidrug-resistant Pseudomonas aeruginosa* (MDRPA) and drug-resistant Streptococcus pneumoniae (DRSP). Several clinical studies also demonstrated the effectiveness of riboflavin/UVA crosslinking as PDT in treatment of bacterial keratitis. [117-119]

1.5 Thesis aims

With increasing resistance of microorganisms to antibiotics, PDT may be a potential alternative in case of therapy resistant infectious keratitis. PDT is based on a photosensitizer activated by light of appropriate wavelength, which leads to generation of singlet oxygen and free radicals, responsible for the cytotoxic effect on microorganisms. Thus, it is important to determine the influence of PDT on human keratocytes and corneal endothelial cells.

The *purpose* of our study was:

- To evaluate the impact of PDT using the photosensitizer Ce6 on viability, apoptosis, proliferation and activation of human keratocytes, in vitro.

- To investigate the impact of PDT using the photosensitizer Ce6 on the secretion of KGF, FGFb, VEGF, HGF and TGFβ1 of human keratocytes, in vitro.

- To determine the impact of PDT using the photosensitizer Ce6 on viability, apoptosis and proliferation of human corneal endothelial cells (HCECs), in vitro.

2 MATERIALS AND METHODS

2.1 Reagents

Dulbecco's Modified Eagle Medium: (Nutrient Mixture F-12 (DMEM/F12)); fetal bovine serum (10%); P/S (1% of 10,000 U/ml penicillin and 10 mg/ml streptomycin); 0.05% trypsin/0.02% ethylenediaminetetra-acetic acid (EDTA) were purchased from PPA Laboratories (Pasching, Austria), Alamar blue from Invitrogen (Karlsruhe, Germany) and propidium iodide from Molecular Probes, Inc. (Eugene, Oregon, USA). Collagenase A, Dispase II and Cell Proliferation ELISA-BrdU (colorimetric) were obtained from Roche Diagnostics (Mannheim, Germany). Fibronectin was from Sigma Chemie (Deisenhofen, Germany). The APO-DIRECT^{TM} Kit and all tissue culture plastics were from BD Biosciences (Heidelberg, Germany). Mouse Anti-Human CD34-FITC was from Biozol (Eching, Germany) and Anti-alpha smooth muscle Actin (α-SMA) antibody (FITC) was from Abcam (Cambridge, USA). KGF was from Blue Gene Biotech (Shanghai, China), FGFb, VEGF, HGF were from RayBiotech (Norcross, USA) and TGFβ1 was from Gen-Probe Incorporated (San Diego, USA). Ce6 was purchased from ORPEGEN Pharma (Heidelberg, Germany).

2.2 Cell culture

2.2.1 Isolation of primary human keratocytes

Human corneas were obtained from the Saarland University Hospital LIONS Eye Bank. Human keratocytes were isolated as described previously. [120,121]

In short, the human corneoscleral buttons were aseptically rinsed in phosphate-buffered saline (PBS) before removal of the endothelium including the Descemet's membrane by sterile surgical disposable scalpel. A central corneal button with epithelium was cut using a 8 mm Barron's trephine and thereafter incubated in culture medium containing 2.4 U/ml Dispase II for 4 hours at 37 °C. In the following, the corneal button was washed with PBS for several times and the already loose corneal epithelium was removed with disposable surgical scalpel. The remaining corneal stroma was incubated in culture medium with 1 mg/ml collagenase A for 8-10 hours at 37 °C. The digested tissue and cells were pipetted three times and centrifuged at 800 g for 7 minutes and finally resuspended in 1 ml culture medium, which consisted of basic medium (DMEM/F12) supplemented with 10% FBS and 1% P/S. The cell suspension was seeded in 6-well plates and the medium was changed 24 hours after seeding.

Medium was changed every 2 to 3 days until keratocytes reached confluence. The cells were subcultured in 25 cm^2 culture flasks after 5 to 10 days following dispersal with 0.05 % trypsin-EDTA for 3 to 5 minutes and the passage 4 to 8 of cells was used for experiments.

2.2.2 Culture of Human Corneal Endothelial Cells (HCECs)

An immortalized human corneal endothelial cell line (HCEC-12, Technical University Dresden, Dresden, Germany) (previously established by SV40 transfection) prepared from a healthy cornea of a 91-year-old Caucasian woman was used for the experiments. Cells were cultured in DMEM/Ham's F12 medium supplemented with 5% FCS and 1% P/S. The culture plates were coated using 20 µg/ml fibronectin. Medium was changed every 2 to 3 days until HCECs reached confluence, and then the cells were subcultured following dispersal with 0.05% trypsin-EDTA for 3 to 5 minutes and passages 4-25 of HCECs were used for experiments.

2.3 Photodynamic Therapy (PDT)

2.3.1 PDT of primary human keratocytes

Human keratocytes were seeded in tissue culture plates and allowed to grow for 48 hours before photodynamic treatment. For viability, apoptosis and proliferation measurements, concentrations of photosensitizer ranged from 50 nM to 64 µM.

Stock solution of Ce6 was prepared with 30 mM concentration. The different concentrations of fresh Ce6 were made using basic medium (DMEM/F12). Cells were washed with PBS once before and after incubation with different concentrations of Ce6 at 37 °C for 30 minutes. Following exposure to the photosensitizer Ce6, Ce6 medium was replaced by basic medium, and then cells were exposed to red light (670 nm) for 13 minutes with a dose of 24 J/cm^2, Ce6 medium was replaced by DMEM/F12 after a washing step with PBS. As control groups, cells were incubated in the dark for 13 minutes after exposure to Ce6. After illumination, the cells were fed with culture medium and cultivated at 37 °C for 24 hours before measurements.

2.3.2 PDT of HCECs

HCECs were seeded in tissue culture plates and allowed to grow for 48 hours before photodynamic treatment. For this experiment, concentrations of the photosensitizer ranged from 0 to 500 nM. Stock solution of chlorin e6 (Ce6) was prepared with 30 mM concentration. The different concentrations of fresh Ce6 solution were made using basic medium (DMEM/F12). Cells were washed with PBS once before and after incubation with different concentrations of Ce6 at 37 °C for 30 minutes. Following exposure to the photosensitizer Ce6, Ce6 medium was replaced by basic medium, and then cells were exposed to red (670 nm) light for 13 minutes. After illumination, the cells were fed with culture medium and let grow at 37 °C for 24 hours before measurements.

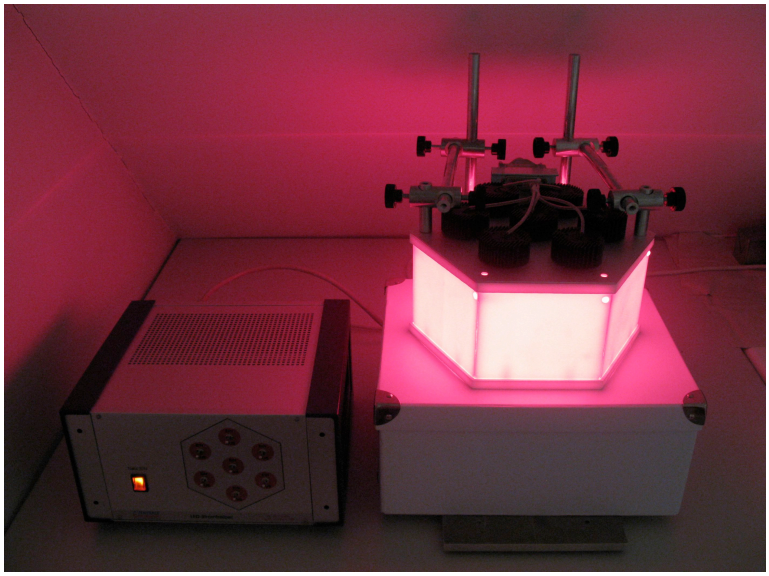

Figure 5. Illumination box (λ = 670 nm, 24 J/cm^2)

2.4 Determination of cell morphology

Twenty-four hours following PDT, morphological changes of keratocytes according to Seitz et al. [120] and HCECs were evaluated by inverted phase contrast microscopy (Eclipse TE2000-S, Nikon, Tokyo, Japan).

2.5 Propidium iodide (PI) immunocytochemisty

Human keratocytes or HCECs were seeded on cover glass in 6-well culture plates with a cell density of 7.5×10^3 cells/cm^2. Twenty-four hours following PDT, cells were fixed on microscope slides with 70% methanol following two washing steps with PBS. Thereafter, the cells were washed with SSC buffer (2×) and incubated with 500 nM of PI solution for 3 minutes at room temperature. The nucleic acid staining of keratocytes and HCECs was analyzed with a fluorescence microscope (Axiovert 200, Carl Zeiss, Jena, Germany).

2.6 Determination of viability (phototoxicity)

Cell viability was evaluated using the Alamar blue assay as follows: Human keratocytes or HCECs were seeded in 24-well cell culture plates at concentration of 7.5×10^3 cells/cm^2 and 1.0×10^5 cells/well, respectively.

At 24 hours after illumination, Alamar blue solution was diluted with culture medium for a final concentration of 10% and 500 µl of this solution was added to each well.

After 3 to 4 hours of incubation, 200 µl of conditioned culture medium from each well was transferred into two wells of 96-well plates. As a negative control, Alamar blue solution was added to a well without cells. Thereafter, all plates were exposed to an excitation wavelength of 560 nm and the emission at 616 nm was recorded using a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences, Wellesley, MA, USA).

2.7 Flow cytometric analysis

To determine the relative number of apoptotic cells (APO-DIRECT^{TM} Kit), human keratocytes or HCECs were seeded in 6-well cell culture plates with a concentration of 7.5×10^3 cells/cm^2 and underwent PDT as described above.

They were harvested at 24 hours following PDT. First, the culture medium was discarded and the cells were trypsinized before centrifugation. Then, the cells were suspended in 1% paraformaldehyd at a concentration of 10×10^5 cells/ml and placed on ice for 30-60 minutes. Thereafter, cells were washed twice with PBS and stored for 30 minutes at -20 °C following adding 1 ml ice cold 70% ethanol. After removing the ethanol carefully by aspiration, fixed cells were resuspended twice in 1 ml wash-buffer. The control cells and the probes were resuspended in 50 µl DNA-labeling-solution (FITC marked dUTP) and the cells were washed twice before resuspending the cell pellet in 500 µl PI/RNase staining buffer (0.3 ml for lower cell amount). Cells were incubated in the dark for at least 30 minutes at room temperature prior to analysis using a FACSCanto flow cytometer (BD Biosciences, Heidelberg, Germany).

2.8 Determination of cell proliferation

The proliferation of the keratocytes or HCECs was determined with the cell proliferation ELISA-BrdU kit, by the measurement of BrdU incorporation in the newly synthesized cellular DNA. Keratocytes were plated at a density of 2×10^3 cells/well and HCECs of 5×10^3 cells/well in a 96-multiwell plate with culture medium of 100 µl, respectively.

PDT was performed as described before with 0, 100 nM and 250 nM Ce6 concentrations. To detect the influence of PDT on the proliferation rate, we tested at 24 hours before PDT, 2 hours and 24 hours after PDT. The test was performed according to the manufacturer's protocol. BrdU was added to the keratocytes or HCECs at the tissue plates and incubated at 37 °C for 4 hours (BrdU incorporation). After removing the culture medium, the cells were fixed with FixDenat, provided with the test kit, followed by the incubation with anti-BrdU-POD, which binds the incorporated DNA. After adding the substrate solution, the immune complexes were detected using an ELISA reader, Model 550 (Bio-Rad Laboratories GmbH, Munich, Germany).

2.9 Expression of CD34 and α-SMA of keatocytes

Cells were seeded in 6-well cell culture plates with a concentration of 4.0×10^3 cells/cm^2 and underwent PDT as described above. They were harvested at 24 hours following PDT with 0 and 100 nM of Ce6 concentration.

First, the culture medium was discarded and the cells were trypsinized and washed with PBS. To demonstrate α-SMA, the cells were incubated with 0.5 ml PERM solution for 10 minutes, and then the cells were washed once with PBS followed by incubation with FITC-conjugated mouse monoclonal antibodies (IgG2a) against human α-SMA (100 μg/10^4 cells) for 30 minutes in dark at room temperature. For CD34, a FITC-conjugated monoclonal antibody (IgG1) was used directly at a concentration of 200 μg/ml followed by an incubation step for 30 minutes in dark at room temperature. To prove specificity of the staining, isotype control experiment for each primary IgG-subtype antibody was performed. In a following step, all cell preparations were washed twice with PBS and analysed using a FACSCanto flow cytometer (BD Biosciences, Heidelberg, Germany), and the evaluation was performed with WinMDI software (Version 2.9).

2.10 Growth factor secretion of keratocytes

Human keratocytes were seeded in 24-well cell culture plates with a concentration of 7.5×10^3 cells/cm^2 and underwent PDT with 0 and 100 nM Ce6 concentrations. They were harvested at five hours and twenty-four hours 24 hours following PDT.

The concentration of KGF, FGFb, VEGF, HGF and TGFβ1 in each well was measured by taking a 100 μl aliquot of the supernatant of the wells. Measurements were performed by ELISA with the following measurement ranges: KGF: 31-2000 pg/ml, FGFb: 102-10000 pg/ml, VEGF: 8-6000 pg/ml, HGF: 2.74-2000 pg/ml and TGFβ1: 30-2000 pg/ml. Measured concentrations below the above values were considered as zero. The growth factor concentrations were quantified by using a human recombinant KGF, FGFb, VEGF, HGF and TGFβ1 as standard. The

measurements were performed exactly following the manufactures' ELISA-protocols. In each well, the concentration of the growth factors in the supernatant was standardized to the cell protein concentration of the respective well. The absorbance was measured at 450 nm (Model 550 Bio-Rad Laboratories GmbH, München, Germany). The experiments were repeated five times using keratocyte cultures of five donor corneas from different patients.

After taking the supernatant for ELISA, the total protein concentration of each well was measured following detachment of the cells with 150 µl CelLytic™ M. Protein quantity was determined according to the method of Bradford, [122] which is based on the formation of a complex between the dye, Brilliant blue G and proteins in solution. The absorbance was measured at 595 nm and the concentrations were quantified using bovine serum albumin (BSA) as standard protein.

2.11 Statistical Analysis

Quantitative data were expressed as means ± standard deviation (mean ± SD). Statistical analysis was performed using the Mann-Whitney U-test for comparisons of the means of two independent groups. To compare data among three or more groups, one-way analysis of variance (ANOVA) followed by the Dunnett's test was performed. $P<0.05$ was considered statistically significant.

3 RESULTS

3.1 Morphology

3.1.1 Keratocyte morphology

Twenty-four hours following application of the photosensitizer Ce6 without exposure to light, cells did not change morphology, and showed a low nuclear cytoplasmic ratio. After a combination of illumination and Ce6 exposure, the impact was dependent on the concentration of Ce6 **(Figure 6)**. With higher Ce6 concentrations, the surviving cells have shown high nuclear-cytoplasmic ratio. With concentrations higher than 2 μM, there were usually only few clusters of cohesive cells left.

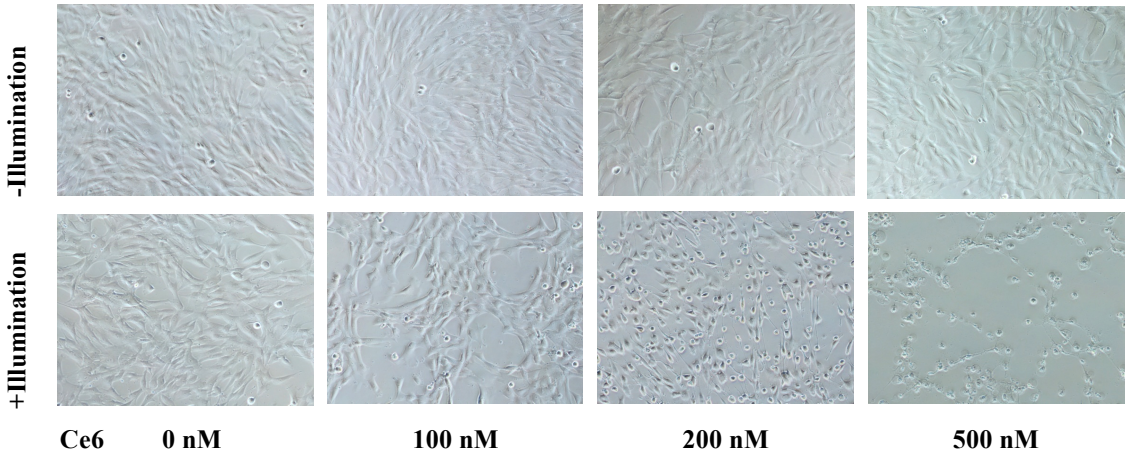

Figure 6. Inverted phase-contrast photomicrographs of human corneal keratocytes 24 hours after exposure to PDT (original magnification 100×). Without illumination, the cell morphology remained unchanged with different concentrations of Ce6, while with illumination, cell shrinkage occurred and keratocytes became detached with increasing concentrations of Ce6.

3.1.2 Morphology of HCECs

Twenty-four hours following application of the photosensitizer chlorin e6 without exposure to light, cells did not change morphology and showed a low nuclear cytoplasmic ratio. After a combination of illumination and Ce6 exposure, the impact was dependent on the concentration of Ce6 **(Figure 7)**. With higher Ce6 concentrations, the surviving cells showed a higher nuclear-cytoplasmic ratio due to shrinkage of the cells.

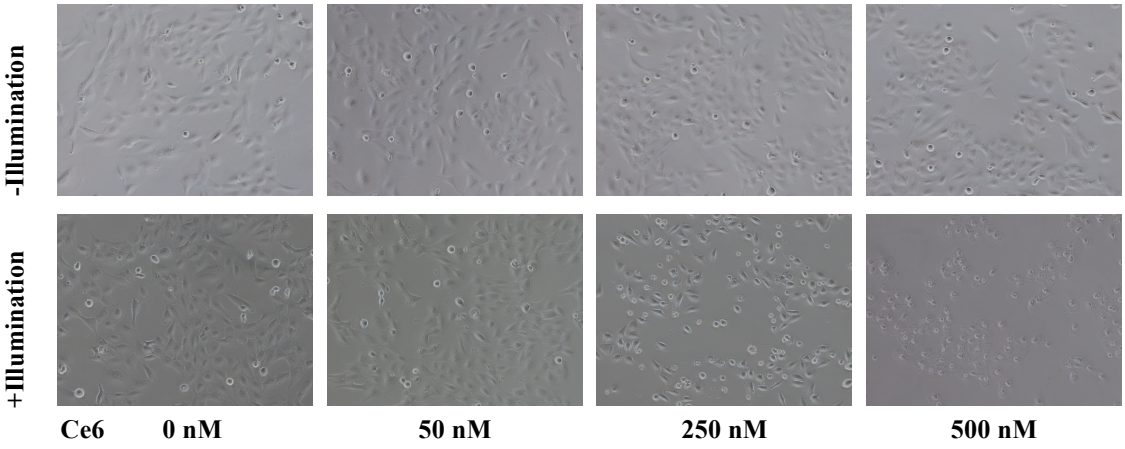

Figure 7. Inverted phase-contrast photomicrographs of HCECs 24 hours after exposure to PDT (original magnification 100×). Without illumination, the cell morphology remained unchanged with different concentrations of Ce6, while with illumination, cell shrinkage occurred and HCECs became detached with increasing concentrations of Ce6.

3.2 Propidium iodide (PI) immunocytochemistry

3.2.1 PI immunocytochemistry of keratocytes

Figure 8 shows PI staining of keratocytes after PDT using different concentrations of Ce6. The prominent red fluorescence of viable cells decreased with increasing concentrations of Ce6, in presence of illumination. With Ce6 concentration of 2 µM, no PI uptake of the keratocytes could be detected.

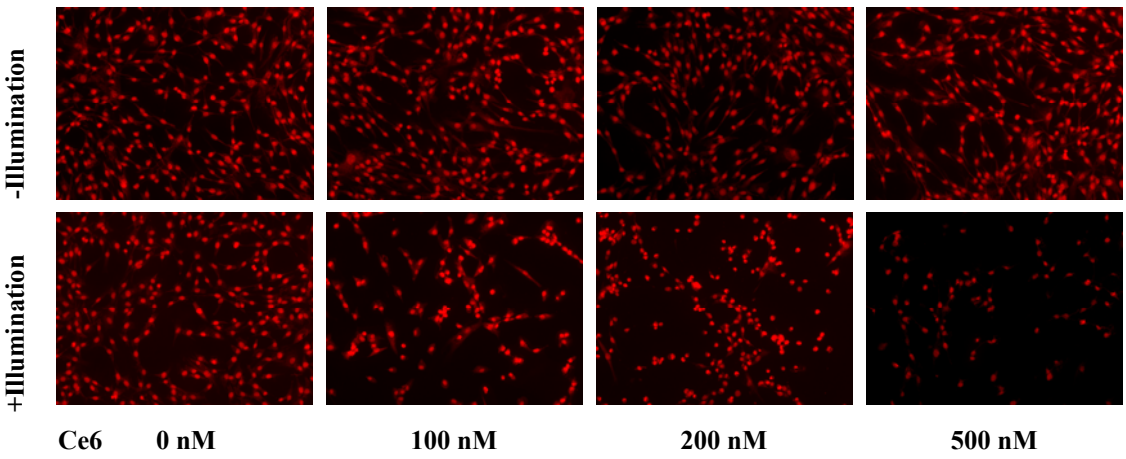

Figure 8. PI staining of human keratocytes 24 hours following PDT. Twenty-four hours after PDT, without illumination, the PI uptake of keratocytes remained unchanged with different concentrations of Ce6, while with illumination, the PI uptake of keratocytes was decreased with increasing concentrations of Ce6.

3.2.2 PI immunocytochemistry of HCECs

Figure 9 shows PI staining of HCECs after PDT, using different concentrations of Ce6. The prominent red fluorescence of viable cells decreased with concentrations of Ce6 higher than 250 nM in the presence of illumination.

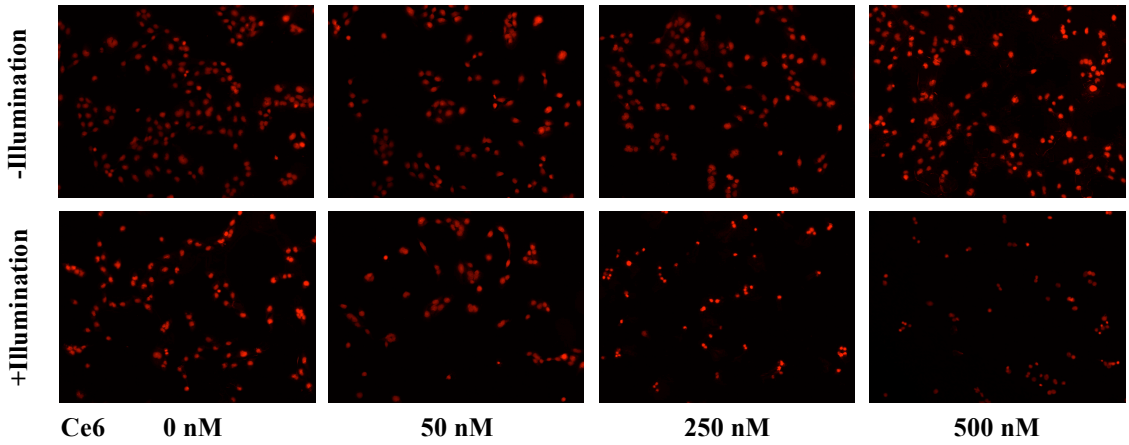

Figure 9. PI staining (red) of HCECs 24 hours following PDT. Twenty-four hours after PDT, without illumination, the PI uptake of HCECs remained unchanged with different concentrations of Ce6, while with illumination, the PI uptake of HCECs was decreased with increasing concentrations of Ce6.

3.3 Viability

3.3.1 Keratocyte viability

Results of the Alamar blue assay are shown in **Figure 10 (n=6)**. Without illumination, no toxicity was observed on keratocytes using different concentrations of the photosensitizer Ce6 up to 2 μM. After dark incubation with 64 μM of Ce6, a decreased viability on keratocytes was obtained ($P=0.02$) compared to control. Following PDT, with Ce6 concentrations higher than 100 nM, keratocyte viability was significantly decreased ($P<0.01$) (Alamar blue assay). Viability has shown even a decreasing trend with increasing concentrations of Ce6 higher than the above value. No survival was observed with concentrations of 2 μM and greater.

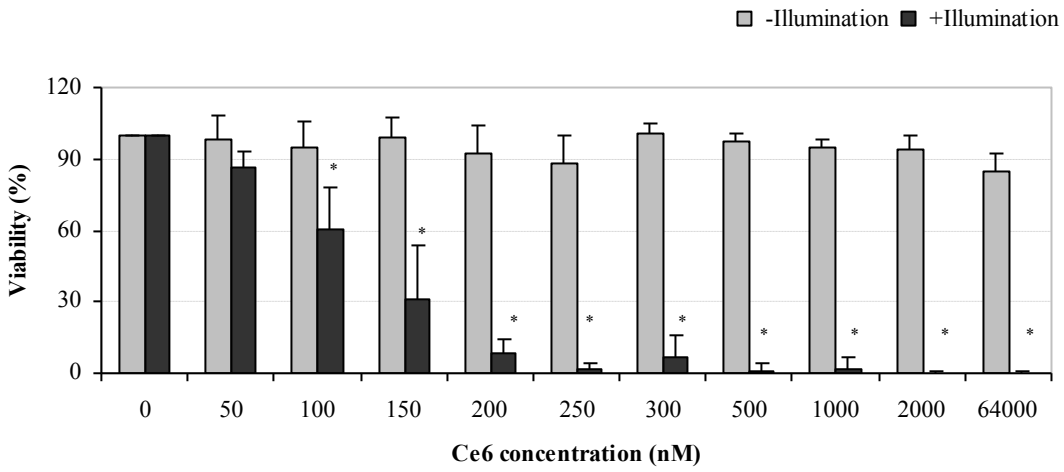

Figure 10. Viability of human keratocytes following PDT using Alamar blue assay. 24 hours after treatment, application of increasing concentrations of Ce6 without illumination did not show an impact on keratocyte viability up to 2 μM, but dark toxicity of keratocytes could be detected at 64 μM concentration of Ce6 without illumination ($^{*}P=0.019$, compared to control). With illumination, with increasing concentrations of Ce6, keratocyte viability decreased significantly from 100 nM concentration of Ce6 ($^{*}P<0.001$, compared to control).

3.3.2 HCECs viability

Results of the Alamar blue assay are shown at **Figure 11 (n=6)**. Without illumination, no toxicity was observed on HCECs using different concentrations of the photosensitizer Ce6 up to 500 nM. Following PDT, with Ce6 concentrations higher than 150 nM, HCECs viability was significantly decreased ($P<0.01$). Viability showed a decreasing trend with increasing concentrations of Ce6 higher than the above value.

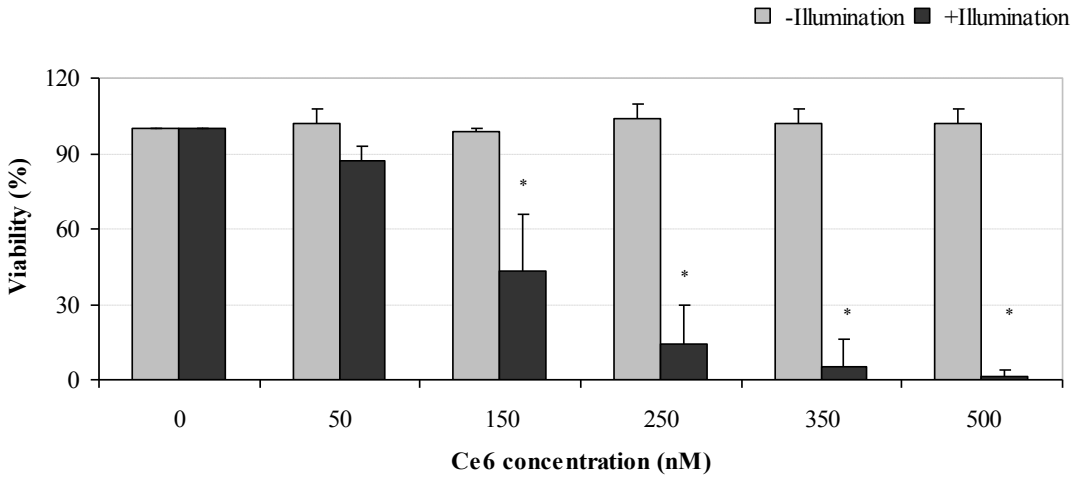

Figure 11. Viability of HCECs following PDT using Alamar blue assay. 24 hours after treatment, application of increasing concentrations of Ce6 without illumination did not show an impact on HCECs. With illumination, with increasing concentrations of Ce6, HCECs viability decreased significantly from concentrations of 150 nM of Ce6 ($^{*}P<0.001$, compared to control).

3.4 Total DNA content and apoptosis

3.4.1 Keratocyte total DNA content

Using the APO-DIRECT^{TM} Kit, illumination without application of the photosensitizer Ce6 or application of Ce6 only had no impact on keratocyte total DNA content **(Figure 12, n=3)**. Using concentrations higher than 250 nM of Ce6, keratocyte total DNA content was significantly decreased ($P<0.01$) following illumination showing a decreasing trend with increasing Ce6 concentrations.

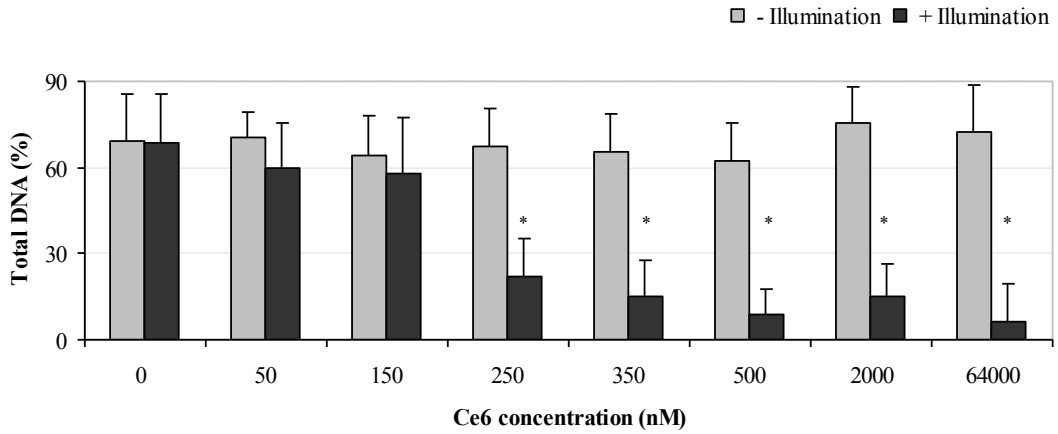

Figure 12. Total DNA content of human keratocytes following PDT using Apo-Direct kit assay. Without illumination, keratocyte total DNA content remained unchanged with different concentrations of Ce6. With illumination, total DNA content of keratocytes decreased significantly from concentrations of 250 nM of Ce6 and higher (*P<0.01, compared to control).

3.4.2 Keratocyte apoptosis

With the APO-DIRECT^{TM} Kit, the apoptotic keratocytes could not be detected after exposure to different Ce6 concentrations in the absence of illumination, or with illumination only. A significant increase of the percentage of apoptotic cells could be detected at 500 nM Ce6 concentration (P<0.01) **(Figure 13, n=3)**.

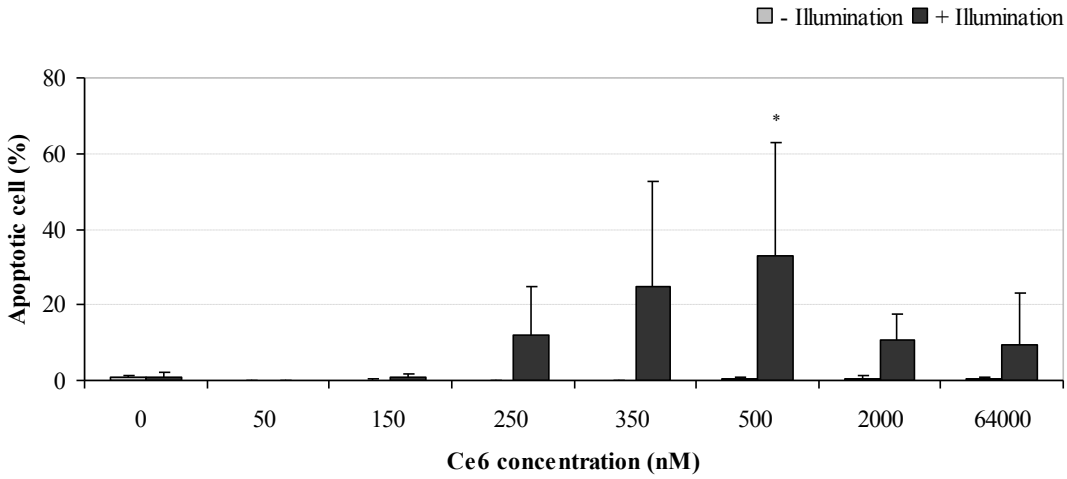

Figure 13. Apoptosis of human keratocytes after PDT using Apo-Direct kit assay. Without illumination, the number of apoptotic keratocytes did not increase significantly using different concentrations of Ce6. With illumination, the number of apoptotic keratocytes increased significantly at 500 nM Ce6 concentration (*P<0.01). At 2 μM and 64 μM concentration, most of the cells were necrotic, and only a small amount of DNA-labeling solution could be incorporated into the non-necrotic cells.

3.4.3 HCECs total DNA content

Using Apo-Direct kit assay, illumination without application of the photosensitizer Ce6 or application of Ce6 only, had no impact on HCECs total DNA content **(Figure 14, n=6)**. Using concentrations higher than 150 nM of Ce6, HCECs total DNA content was decreased (P<0.05) following illumination. Furthermore, a decreasing trend was shown with increasing Ce6 concentrations.

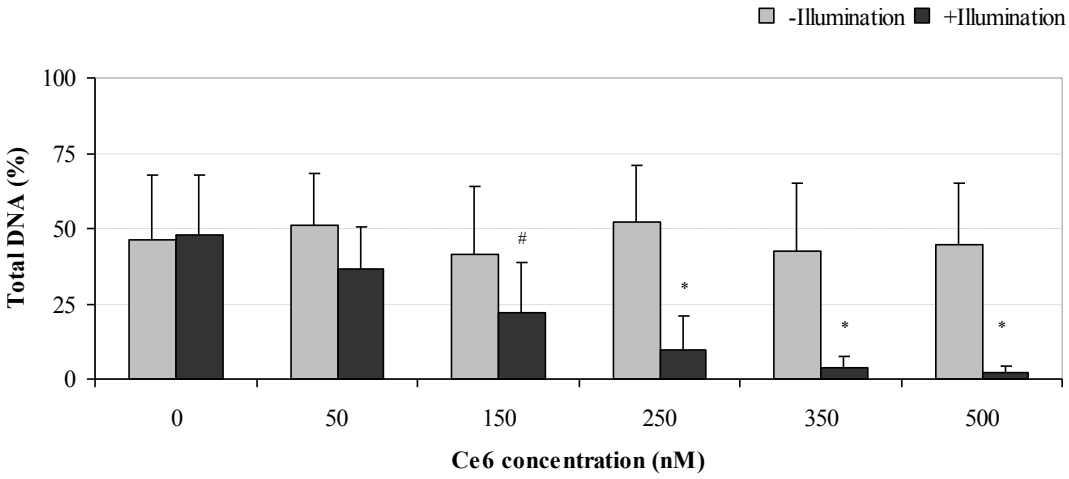

Figure 14. Total DNA content of HCECs following PDT using Apo-Direct kit assay. Without illumination, HCECs total DNA content remained unchanged with different concentrations of Ce6. With illumination, total DNA content of HCECs decreased from concentrations higher than 150 nM of Ce6 ($^{\#}P = 0.043$, $*P < 0.01$, compared to control).

3.4.4 HCECs apoptosis

With Apo-Direct kit assay, the apoptotic HCECs could not be detected after exposure to different Ce6 concentrations in the absence of illumination, or with illumination only. An increase of the percentage of apoptotic cells could, however, be detected from concentrations higher than 250 nM of Ce6 ($P < 0.01$) **(Figure 15, n=6)**.

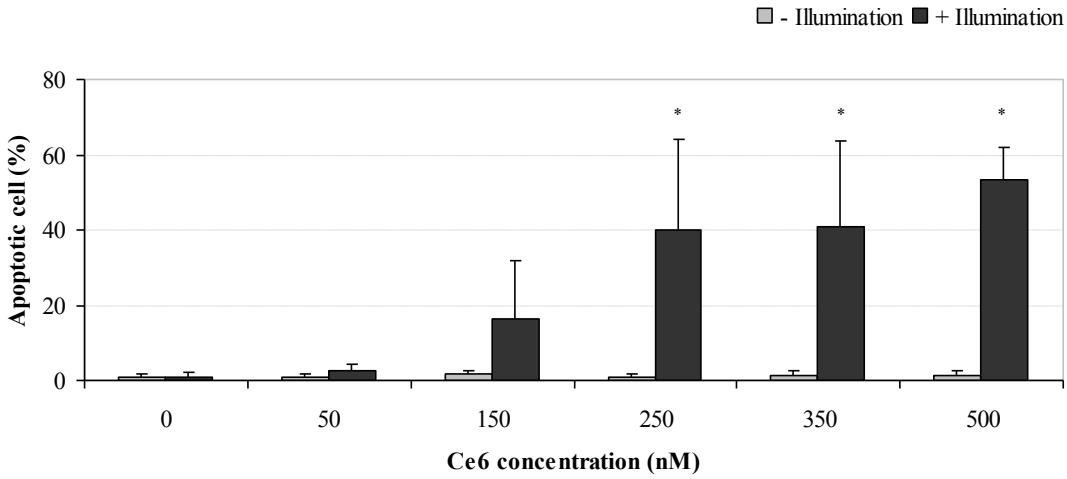

Figure 15. Percentage of apoptotic HCECs after PDT using Apo-Direct kit assay. Without illumination, the number of apoptotic HCECs did not increase significantly using different concentrations of Ce6. With illumination, the number of apoptotic HCECs increased significantly from 250 nM Ce6 concentration (*P<0.01, compared to control).

3.5 Proliferation

3.5.1 Keratocyte proliferation

Two hours after PDT, there was no significant change in keratocyte proliferation using different Ce6 concentrations, with or without illumination. Twenty-four hours following PDT, the proliferation of cells was inhibited significantly with 250 nM Ce6 concentration (P=0.01) compared to control **(Figure 16, n=4)**.

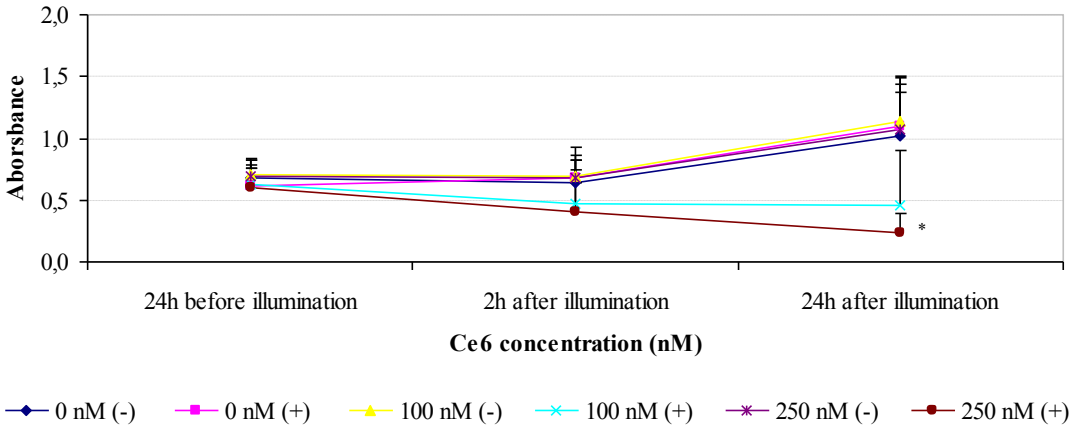

Figure 16. Proliferation of human keratocytes following PDT using BrdU Cell Proliferation Assay Kit. 24 hours after illumination, the proliferation of keratocytes was significantly inhibited using 250 nM Ce6 concentration (*P=0.013) compared to control, while it was unchanged 2 hours after PDT. 24h before illumination group was tested as a starting point in cell growth under the same condition without PDT treatment. (-) as without illumination and (+) as with illumination.

3.5.2 HCECs proliferation

Two hours after PDT, there was no significant change in HCECs proliferation using different Ce6 concentrations without illumination, or illumination only. Two hours following PDT the proliferation of cells was inhibited significantly at 250 nM Ce6 concentration ($P<0.01$) **(Figure 17, n=6),** and twenty-four hours after PDT at 100 nM ($P=0.019$) and 250 nM ($P<0.01$) concentrations of Ce6 compared to control.

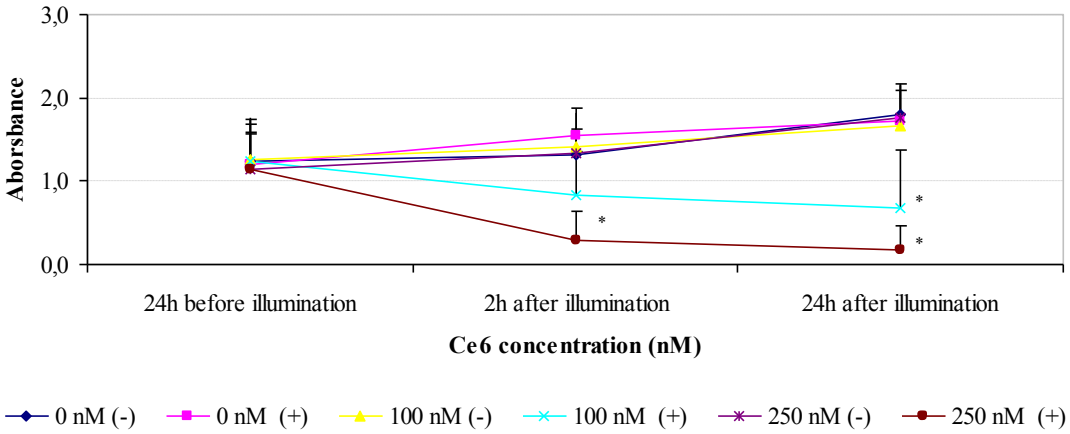

Figure 17. Proliferation of HCECs following PDT using BrdU Cell Proliferation Assay Kit. 24 hours after illumination for 13 min, the proliferation of HCECs was significantly inhibited using 100 nM (*P=0.019) and 250 nM (*P<0.01) Ce6 concentration, and it was decreased at 250 nM Ce6 concentration (*P<0.01) 2 hours after illumination compared to control. (-) Without illumination, (+) With illumination for 13 min.

3.6 CD34 and α-SMA expression of keratocytes

CD34 and α-SMA expression of keratocytes 24 hours following PDT are summarized in **Figures 18 and 19 (n=8)**. Using Ce6 or illumination only, CD34 and α-SMA expression of the cells did not change significantly. Twenty-four hours after PDT, the expression of CD34 has shown an increasing trend with increasing concentration of Ce6. The percentage of α-SMA positive keratocytes decreased significantly (P=0.02) compared to controls at 250 nM concentration of Ce6 following illumination.

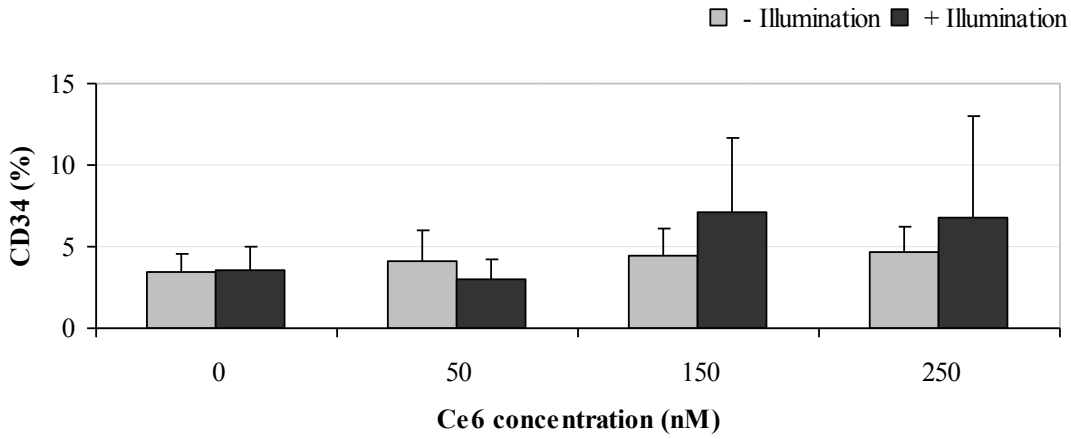

Figure 18. CD34 expressions of human keratocytes 24 hours after PDT. Following illumination, the percentage of CD34 positive keratocytes has shown an increasing trend with increasing Ce6 concentrations, however, there was no significant difference compared to controls.

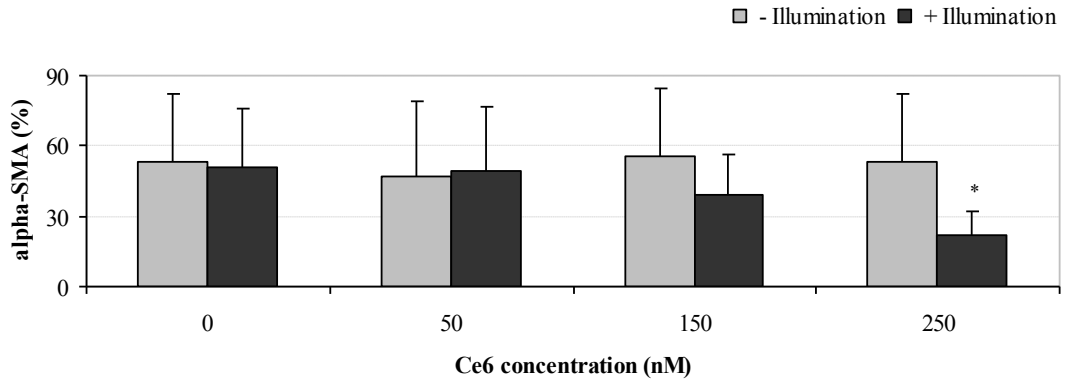

Figure 19. α-SMA expression of human keratocytes 24 hours after PDT. Following illumination, the percentage of α-SMA positive keratocytes has shown a decreasing trend with increasing Ce6 concentrations. At 250 nM Ce6 concentration, the percentage of α-SMA positive cells decreased significantly following illumination compared to controls (*P=0.02).

3.7 Growth factor secretion of keratocytes

Five hours after PDT, the concentrations of KGF, FGFb, VEGF, HGF and TGFβ1 are summarized at **Table 2 (n=5)**. Following PDT, using 100 nM Ce6 and illumination, the mean FGFb concentration was 5.26 ± 1.49 pg/µg protein in the supernatant of cultured keratocytes. This was significantly higher than FGFb concentration at untreated keratocyte cultures ($P<0.01$). The FGFb secretion also increased using illumination only compared to control ($P<0.01$). Five hours following PDT, the secretion of HGF was significantly lower than in untreated controls ($P=0.01$) (**Figure 20, n=5**). In addition, HGF concentration was significantly lower following Ce6 exposure and illumination, as compared to illumination alone ($P=0.03$).

We could not detect changes in the secretion of KGF and VEGF of keratocytes with or without illumination five hours following PDT. FGFb, HGF, KGF, VEGF concentrations using only Ce6 or illumination did not differ significantly from untreated controls. The secretion of TGFβ1 was under the measurement limit in the treated and untreated cell cultures five hours after PDT.

Groups	Control	Ce6	Illumination	Ce6 + Illumination	* p-value	** p-value	*** p-value
KGF	1.33 ± 0.42	1.20 ± 0.21	1.38 ± 0.32	1.40 ± 0.37	0.51	0.49	0.88
FGFb	3.47 ± 0.94	4.06 ± 1.02	4.06 ± 1.22	5.26 ± 1.49	**< 0.01**	**< 0.01**	0.24
VEGF	1.38 ± 0.76	1.23 ± 0.42	1.10 ± 0.58	1.05 ± 0.96	0.37	0.27	0.75
HGF	0.43 ± 0.23	0.39 ± 0.27	0.39 ± 0.27	0.24 ± 0.25	0.35	**0.01**	**0.03**
TGFβ1	n.d.	n.d.	n.d.	n.d.	n/a	n/a	n/a

Table 2. Concentration (pg/µg protein) of different growth factors in keratocyte cultures five hours after PDT. Values indicate mean ± SD.

* p-values indicate the difference between "Control" versus "Illumination alone" groups (Mann-Whitney U test).

** p-values indicate the difference between "Control" versus "Ce6 + Illumination" groups (Mann-Whitney U test).

*** p-values indicate the difference between "Illumination alone" versus "Ce6 + Illumination" groups (Mann-Whitney U test).

Significant values are shown in bold.

n.d. = not detectable and n/a = not applicable.

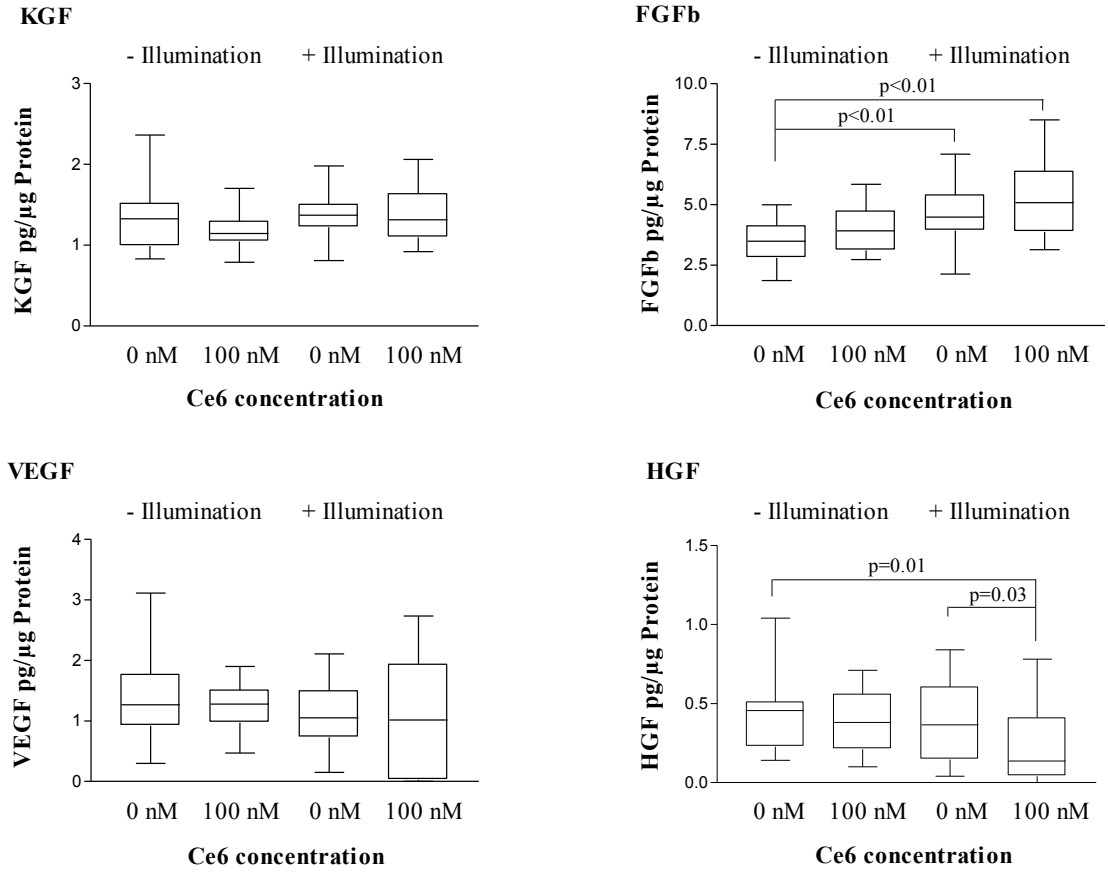

Figure 20. Concentration of different growth factors 5 hours following PDT. Significant differences are indicated.

Table 3 and **Figure 21 (n=5)** display concentrations of KGF, FGFb, VEGF, HGF and TGFβ1 24 hours after PDT. *Twenty-four hours* following PDT, the secretion of KGF was significantly lower than in untreated control ($P<0.01$). The KGF expression also decreased with illumination only compared to control cells ($P<0.01$).

The secretion of FGFb, VEGF, HGF and TGFβ1 did not change significantly compared to untreated control keratocyte cultures for this time point. However, TGFβ1 concentration was significantly lower following illumination only, than after Ce6 exposure and illumination ($P=0.01$). Similarly, VEGF concentration was significantly lower after illumination, as compared to that following Ce6 exposure and illumination ($P<0.01$).

Groups	Control	Ce6	Illumination	Ce6 + Illumination	* p-value	** p-value	*** p-value
KGF	6.57 ± 0.57	6.85 ± 0.56	5.37 ± 0.55	5.67 ± 0.63	**<0.01**	**<0.01**	0.23
FGFb	5.51 ± 3.83	5.88 ± 2.79	4.72 ± 2.92	4.18 ± 1.74	0.39	0.26	0.64
VEGF	7.74 ± 4.21	7.38 ± 3.64	5.95 ± 3.35	9.01 ± 3.43	0.21	0.16	**<0.01**
HGF	1.59 ± 1.52	1.34 ± 1.08	1.72 ± 1.69	1.61 ± 1.36	0.99	0.61	0.89
TGFβ1	3.40 ± 1.53	3.21 ± 1.11	2.83 ± 1.30	3.76 ± 1.17	0.19	0.32	**0.01**

Table 3. Concentration (pg/μg protein) of different growth factors in keratocyte cultures twenty-four hours after PDT. Values indicate mean ± SD.

* p-values indicate the difference between "Control" versus "Illumination alone" groups (Mann-Whitney U test).

** p-values indicate the difference between "Control" versus " Ce6 + Illumination" groups (Mann-Whitney U test).

*** p-values indicate the difference between "Illumination alone" versus "Ce6 + Illumination" groups (Mann-Whitney U test).

Significant values are shown in bold.

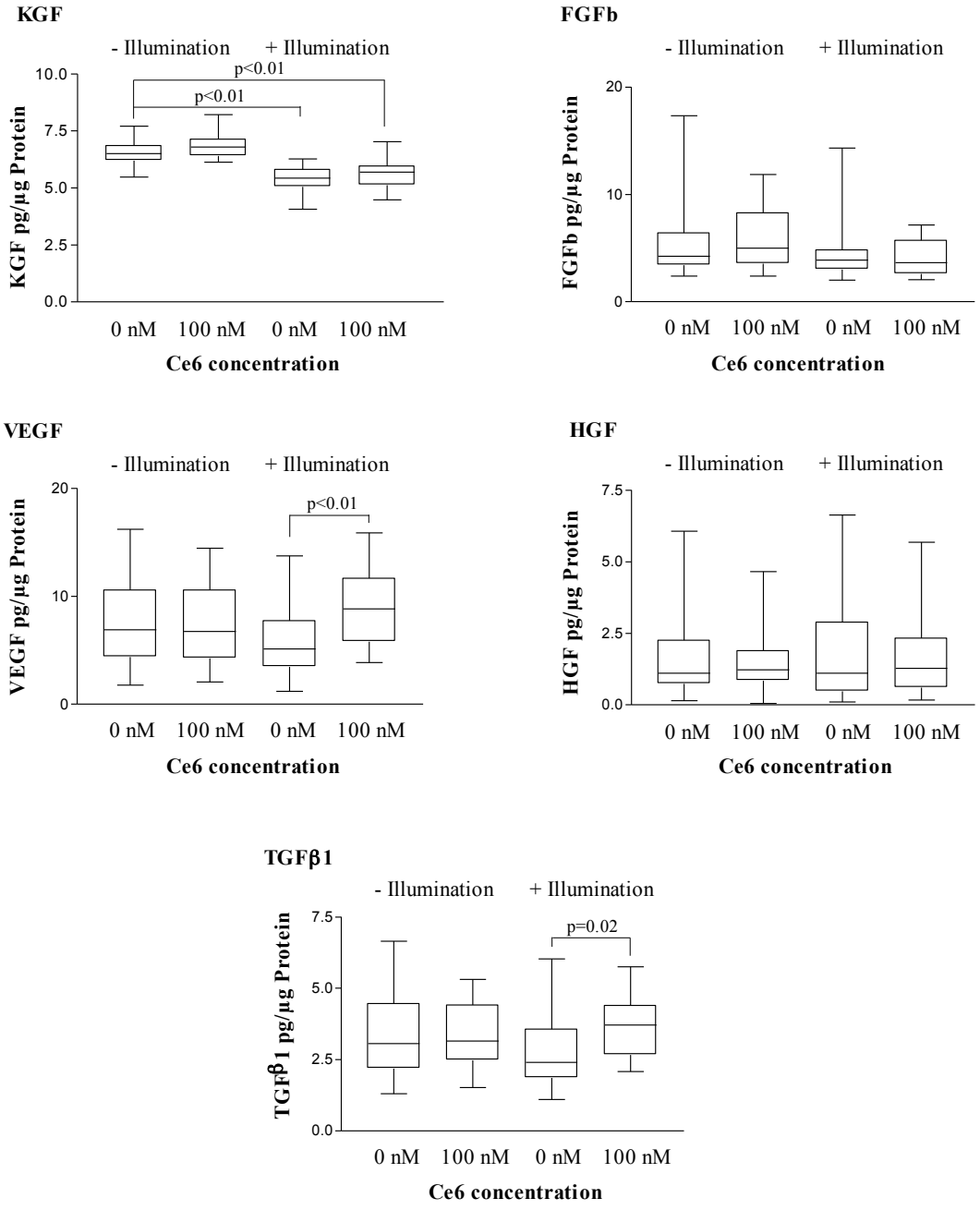

Figure 21. Concentration of different growth factors 24 hours following PDT. Significant differences are indicated.

4 DISCUSSION

4.1 Impact of PDT on viability, apoptosis, proliferation and activation of human keratocytes

In recent years, PDT became an important treatment modality for therapy resistant infectious keratitis. The ORS produced during PDT result in apoptosis or even necrosis of the cells and tissues. The possible adverse effects on normal mammalian cells or tissues are the limiting factors of the use of PDT. Nevertheless, PDT may also result in activation of keratocytes or even modification of the local immune response to pathogens. This is the first study illustrating the viability, apoptosis and proliferation of human keratocytes in vitro following PDT using a porphyrin derivative, in order to evaluate the "tolerance" of normal keratocytes to PDT. Ce6, with its high photosensitizing efficacy may be a photosensitizer of choice in localized infections for the future. [92]

In the present in vitro study we demonstrated that PDT using the photosensitizer Ce6 reduces the viability of keratocytes in a dose-dependent manner and induces apoptosis of the cells. We could determine that the cell cohesion was reduced and the clusters of cells became smaller with increasing concentrations of Ce6 following PDT. Cell viability was decreased from 100 nM Ce6 concentration using Alamar blue assay, and total DNA content of the cells decreased from 250 nM Ce6 concentration using the APO-DIRECT^{TM} Kit following illumination. Accordingly, the percentage of apoptotic

cells also increased from 250 nM concentration of Ce6 after illumination, and a significant increase at 500 nM concentration of Ce6 was reached.

Corneal collagen crosslinking (CLX) uses riboflavin as photosensitizer and UVA light for illumination/excitation. CLX increases effectively the biomechanical rigidity of the cornea in order to delay or stop the progression of keratoconus. [123] The potential of riboflavin to heal bacterial or acanthamoeba keratitis has been shown recently. [119] Using collagen crosslinking, Wollensak et al. [124] could detect similar impact on keratocytes in vitro with an abrupt cytotoxic effect (Trypan blue and Yopro-fluorescence staining).

The proliferation of keratocytes was decreased at 250 nM Ce6 concentration 24 hours after PDT, in our study. In contrast, at a later time point, 6 months following corneal crosslinking in a histological study, a significant increase in keratocyte proliferation could be shown using Ki-67 staining. [125] In our opinion, the induced keratocyte proliferation detected 6 months after CLX may be part of the long-term repair mechanisms after the treatment.

The most conspicuous finding of our study is that using subcytotoxic concentrations of the photosensitizer Ce6, CD34 expression will be slightly upregulated and α-SMA expression downregulated in keratocytes 24 hours following PDT. With other words, hematopoietic stem cell activation will be tendentially promoted, myofibroblastic transformation inhibited through PDT.

CD34 is a surface glycophosphoprotein expressed on lymphohemotopoietic stem cells, vascular endothelial cells, embryonic fibroblasts and fibroblast-like dendritic cells in connective tissues. [126] Sosnová et al. [127] reported that the larger population of bone marrow derived leukocytes in the corneal stroma are CD34 positive. Studies of Dua et

al. [128] confirmed that CD34 positive keratocytes are multipotent hemopoetic stem cells.

Increased α-SMA expression is detected in myofibroblasts, not in keratocytes. [129] Corneal myofibroblasts are determinant in corneal wound healing and contraction. Recent studies have shown that corneal myofibroblasts also have an important role in bacterial and viral clearance by the expression of toll-like receptor (TLR), which is a receptor for lipopolysaccharides produced by bacteria. [130] It was also described previously, that CD34 expression in human keratocytes is downregulated during myofibroblast differentiation. [131] The role of CD34 positive hemopoetic stem cells may be to replenish tissue resident macrophages and plasmacytoid dendritic cells. [132,133] Studies on the function of CD34 suggest that it may also play a role in cytoadhesion, and signalling related to differentiation and proliferation. [134]

4.2 Growth factor secretion of human keratocytes following PDT

PDT using Ce6 decreases viability and proliferation, suppresses myofibroblastic transformation and triggers apoptosis of human keratocytes, which may also lead to inflammatory cell response, in vitro. [135], [136] The impact of PDT on secretion of growth factors of human keratocytes was analyzed in the present study.

PDT using subcytotoxic concentration of Ce6 increases FGFb and decreases HGF secretion of keratocytes 5 hours after treatment. Twenty-four hours following PDT concentration of both growth factors normalizes in culture, however, the concentration of KGF decreases. A limitation of our study is that using ELISA, only secreted growth factors were measured and total protein or mRNA analysis was not performed.

It is known, that mRNA coding for TGFβ1, FGFb, KGF, and HGF are represented in corneal cells. [137-139] These growth factors are associated to paracrine interactions between different cell types of the cornea, especially with an impact on corneal epithelial cells.

FGFb promotes angiogenesis, cell proliferation and migration. PDT increased the expression of FGFb five hours after exposure to 100 nM concentration of Ce6 combined with 370 nm light, and with that, may induce angiogenesis in the cornea and keratocyte proliferation acting in an autocrine manner. Furthermore FGFb induces the differentiation of keratocytes into a fibroblastic phenotype. [129] Interestingly, Ley et al. [140] also found increased FGFb secretion in the cornea of Monodelphis domestica after 360 nm ultraviolet light.

HGF is known to delay the process of reepithelialization. The decrease of HGF five

hours after PDT may result in decreased proliferation and migration of both epithelial cells and keratocytes acting as a paracrine and autocrine regulator. [141] HGF also induces myofibroblastic transformation of keratocytes. With decreased HGF secretion, myofibroblastic tranformation may be inhibited after PDT. In accordance with that, we have also found decreased alpha-smooth actin expression of keratocytes using 250 nM concentration of Ce6, following PDT. Lee et al. [142] reported on the inhibitory effect of HGF on apoptosis of bovine pulmonary artery endothelial cells. Our current findings indicate that the decreased HGF secretion of keratocytes in cell culture is too low to prevent them from apoptosis five hours after PDT.

Some authors reported on increased KGF mRNA expression in wounded mouse corneas. [143,144] In contrast, our study could not determine a significant impact of PDT on KGF secretion 5 hours following treatment, but its secretion decreased after 24 hours. KGF was reported to accelerate corneal epithelial wound healing in vivo, [145] which could be delayed 24 hours after PDT, with decreased KGF secretion.

Interestingly, following a slight decrease of VEGF secretion 5 hours after PDT, its concentration increased 24 hours after treatment. These results indicate that PDT may promote corneal haem- and lymphangiogenesis through the vascular endothelial growth factor one day following treatment. [146]

It has been previously reported, that TGFβ1 induces CD34 expression of keratocytes in cell culture. [131] TGFβ1 also promoted myofibroblastic transformation of keratocytes following corneal injury. [147] Honma et al. [148] have reported that TGFβ1 and TGFβ2 also inhibit the promotion of KGF and HGF dependent cell proliferation. Five hours following PDT, we could not detect significant secretion of TGFβ1 and 24 hours after PDT its secretion was only slightly increased.

This is also in accordance with our previous measurements: at 100 nM Ce6

concentration, CD34 and α-SMA expression of keratocytes did not change significantly after 24 hours. Our study could not determine a decisive role of TGFβ1 secretion on CD34 expression or myofibroblastic transformation of keratocytes 5 and 24 hours after PDT.

Interestingly, using illumination or the photosensitizer Ce6 separately, we did not detect changes in viability, apoptosis, CD34 or alpha smooth-actin expression of keratocytes, at our previous measurements. [149,150] In contrast, FGFb secretion 5 hours following the use of illumination only and KGF secretion 24 hours after illumination only significantly differed from controls. In addition, using illumination, 5 hours after treatment, HGF secretion was significantly decreased at 100 nM Ce6 concentration compared to illumination alone, and after 24 hours, VEGF and TGFβ1 secretion was significantly increased. It is known from previous studies that the use of a photosensitizer or illumination separately may have an impact on growth factor secretion of the cells; however, the detailed molecular mechanisms are still to be clarified. [151]

4.3 Impact of PDT on viability, apoptosis and proliferation of HCECs

HCECs located as a single cell layer at the posterior surface of the cornea play a crucial role in maintaining corneal transparency. Therefore, using PDT in keratitis, cytotoxic effects on HCECs should be as small as possible. Corneal endothelial cells are also much more sensible for stress than epithelial cells [152] or keratocytes [153,154] or other cell types. [155,156]

The last few years Ce6 is widely used as second-generation photosensitizer. The main advantages of Ce6 are its low toxicity, easy synthesis and production, fast and sufficiently selective accumulation in target tissue and high photosensitizing efficacy. [92] Penetration depth of the photosensitizer Ce6 in the cornea depends on photosensitizer concentration, exposition time and room temperature. After removal of the corneal epithelium using 450 µM of Ce6 for 15 minutes at 35 ˚C, Ce6 penetrates until 400 µm stromal depth.[157]

In the present study we demonstrated that PDT reduces the viability of HCECs in a dose-dependent manner and induces apoptosis of the cells. Alamar blue assay has shown a significant cytotoxic effect on corneal endothelial cells after application of at least 150 nM concentration of Ce6 and 13 minutes of red-light illumination. There was a significant increase in the percentage of apoptotic HCECs using at least 250 nM Ce6 concentration and illumination.

In addition, we also determined that proliferation of HCECs was inhibited 2 and 24 hours after exposure to illumination using 250 nM concentration of Ce6. As our HCEC line was an immortalized human corneal endothelial cell line, [158] with a

different manner of proliferative capacity compared to primary HCECs, the impact of our study is limited. Nevertheless, we hypothesized that the decreased cell proliferation may be in relation to the significant HCEC loss through apoptosis at this concentration.

Corneal collagen crosslinking is a new technique to increase effectively the biomechanical rigidity of the cornea and to delay or stop the progression of keratoconus. [123,159] Collagen crosslinking may also be interpreted as a special type of PDT, which uses riboflavin as photosensitizer and ultraviolet-A (UVA) light (370 nm) for illumination. The cytotoxic effect on corneal endothelial cells is already known by the use of relatively high UVA irradiance (4 mW/cm^2) or a combined UVA (0.35 mW/cm^2) treatment and the use of 0.025% riboflavin solution (500 μM) in vitro. [160]

An in vivo study of rabbit corneas [161] has also demonstrated a cytotoxic effect on corneal endothelium using the standard surface irradiance of 3.0 mW/cm^2 combined with an application of 0.1% riboflavin-5-phosphate and 20% dextran T-50 on the cornea. In our study, we observed a significant cytotoxic effect on human corneal endothelial cells using an irradiance of 31 mW/ cm^2 (670 nm) for 13 minutes combined with 150 nM concentration of Ce6 in vitro. The difference may originate from the distinct photodynamic efficacy of both photosensitizers.

4.4 Conclusions and outlook to the future

With increasing resistance of microorganisms to antibiotics, alternative therapeutic options are required in the treatment of infectious keratitis. Some authors claim that "photodynamic therapy" (PDT) is one potential therapeutic option. [97] Since the first case report of Micelli Ferrari et al. [162] in 2009 on succesful treatment of Eschericia coli keratitis using riboflavin-UVA-crosslinking therapy (as PDT), the number of patients treated using his therapeutic option increases. [97]

Lethal photosensitization is a process by which a photosensitizer is activated by light of appropriate wavelenght resulting in the production of cytotoxic reactive oxygen species, which then kill the target cell. However, this also means that commensal bacteria and host tissues could be adversely affected.

In our work we determined that:

- PDT using Ce6 decreases viability and proliferation, suppresses myofibroblastic transformation and triggers apoptosis of human keratocytes, in vitro.
- PDT triggers FGFb and inhibits HGF secretion of human keratocytes 5 hours and inhibits KGF secretion 24 hours following treatment.
- PDT using Ce6 decreases viability and proliferation, and also triggers apoptosis of HCECs, in vitro.

In human keratocyte and endothelial cell cultures - using a standard irradiation dose - we may determine a treshold cytotoxic concentration for the photosensitizer (increased apoptotosis). [124,160] Using the same standard irradiation dose, in order to reach 90% killing rate of SA, SE or PA in vitro, application of about 400 x of the cytotoxic treshold concentration of the photosensitiser is necessary. [63] This condition

occurs using the photosensitizer riboflavin and UVA light illumination, [63,124,160] or even with porphyrins as photosensitizer and red light excitation. [114] Therefore, it is important to develop a treatment method that could specifically target the pathogenic organisms without causing any adverse effects on the host tissue.

For PDT, one method of achieving this is to conjugate the photosensitizer to an antibody raised directly against microorganisms. Photosensitizers that have been up to now conjugated to antibodies include porphyrins, sulfonated aluminium phthalocyanine and hematoporphyrin. [163] As an example, polycationic conjugates between poly-L-lysine chains and the porphyrin Ce6 are particularly suited to fight both Gram positive or negative bacteria and also funghi. [164]

With antibody-targeted photosensitisation it was possible to kill 99% of MRSA using only 2.5 µg/ml of the photosensitizer Ce6, which concentration is only 18 x above the cytotoxic treshold of keratocytes. [97,163] In addition, the antibody-targeted photosensitiser with specific bindig to pathogens may cause even less damage to human cells. [164]

Antibody-targeted photosensitisation may open a new horison in the treatment of infectious keratitis. Development of aspecific or even specific antibody-photosensitizer conjugates may offer a further treatment alternative for therapy resistant cases.

5 REFERENCES

1. Kanski JJ, Bowling B, Nischal K, Pearson A. Clinical ophthalmology: a systematic approach. Edinburgh; New York: Elsevier/Saunders 2011;7th edition:p167-235.
2. Jones DB. Decision-making in the management of microbial keratitis. Ophthalmology 1981; 88:814-820.
3. Klyce SD. Electrical profiles in the corneal epithelium. J Physiol 1972;226:407-429.
4. Secker GA, Daniels JT. Limbal epithelial stem cells of the cornea. StemBook: ed The Stem Cell Research Community, StemBook 2009:p1-18.
5. Schlotzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. Exp Eye Res 2005;81:247-264.
6. Kobayashi A, Sugiyama K. In vivo corneal confocal microscopic findings of palisades of Vogt and its underlying limbal stroma. Cornea 2005;24:435-437.
7. Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature 1971;229:560-561.
8. Dua HS, Saini JS, Azuara-Blanco A, Gupta P. Limbal stem cell deficiency: concept, aetiology, clinical presentation, diagnosis and management. Indian J Ophthalmol 2000;48: 83-92.
9. Dua HS, Joseph A, Shanmuganathan VA, Jones RE. Stem cell differentiation and the effects of deficiency. Eye (Lond) 2003;17:877-885.
10. Wilson SE, Hong JW. Bowman's layer structure and function: critical or dispensable to corneal function? A hypothesis. Cornea 2000;19:417-420.
11. Maurice DM. The structure and transparency of the cornea. J Physiol 1957;136:263-286.
12. Meek KM, Boote C. The organization of collagen in the corneal stroma. Exp Eye Res 2004;78:503-512.
13. Funderburgh JL, Mann MM, Funderburgh ML. Keratocyte phenotype mediates proteoglycan

structure: a role for fibroblasts in corneal fibrosis. J Biol Chem 2003;278:45629-45637.

14. Portis JM, Stamper RL, Spencer WH, Webster RG, Jr. The corneal endothelium and Descemet's membrane in the iridocorneal endothelial syndrome. Trans Am Ophthalmol Soc 1985;83:316-331.
15. Maurice DM. The location of the fluid pump in the cornea. J Physiol 1972;221:43-54.
16. Bourne WM. Clinical estimation of corneal endothelial pump function. Trans Am Ophthalmol Soc 1998;96:229-239; discussion 239-242.
17. Srinivas SP. Dynamic regulation of barrier integrity of the corneal endothelium. Optom Vis Sci 2010;87:E239-254.
18. Laing RA, Sanstrom MM, Berrospi AR, Leibowitz HM. Changes in the corneal endothelium as a function of age. Exp Eye Res 1976;22:587-594.
19. Gagnon MM, Boisjoly HM, Brunette I, Charest M, Amyot M. Corneal endothelial cell density in glaucoma. Cornea 1997;16:314-318.
20. Pechmeja J, Guinguet J, Colin J, Binder PS. Severe endothelial cell loss with anterior chamber phakic intraocular lenses. J Cataract Refract Surg 2012;38:1288-1292.
21. Seal DV, Kirkness CM, Bennett HG, Peterson M. Population-based cohort study of microbial keratitis in Scotland: incidence and features. Cont Lens Anterior Eye 1999;22:49-57.
22. Lam DS, Houang E, Fan DS, Lyon D, Seal D, Wong E. Incidence and risk factors for microbial keratitis in Hong Kong: comparison with Europe and North America. Eye (Lond) 2002;16:608-618.
23. Jeng BH, Gritz DC, Kumar AB, Holsclaw DS, Porco TC, Smith SD, Witcher JP, Margolis TP, Wong IG. Epidemiology of ulcerative keratitis in Northern California. Arch Ophthalmol 2010;128:1022-1028.
24. Gonzales CA, Srinivasan M, Whitcher JP, Smolin G. Incidence of corneal ulceration in Madurai district, South India. Ophthalmic Epidemiol 1996;3:159-166.
25. Upadhyay M, Karmacharya P, Koirala S, Shah D, Shakya S, Shrestha J, Bajracharya H, Gurung C, Whitcher J. The Bhaktapur eye study: ocular trauma and antibiotic prophylaxis for the prevention of corneal ulceration in Nepal. Br J Ophthalmol 2001;85:388-392.
26. Xie L, Zhong W, Shi W, Sun S. Spectrum of fungal keratitis in north China. Ophthalmology

2006;113:1943-1948.

27. Bharathi MJ, Ramakrishnan R, Meenakshi R, Padmavathy S, Shivakumar C, Srinivasan M. Microbial keratitis in South India: influence of risk factors, climate, and geographical variation. Ophthalmic Epidemiol 2007;14:61-69.
28. Titiyal JS, Negi S, Anand A, Tandon R, Sharma N, Vajpayee RB. Risk factors for perforation in microbial corneal ulcers in north India. Br J Ophthalmol 2006;90:686-689.
29. Keay L, Edwards K, Naduvilath T, Taylor HR, Snibson GR, Forde K, Stapleton F. Microbial keratitis predisposing factors and morbidity. Ophthalmology 2006;113:109-116.
30. Watt K, Swarbrick HA. Microbial keratitis in overnight orthokeratology: review of the first 50 cases. Eye Contact Lens 2005;31:201-208.
31. Das S, Constantinou M, Daniell M, Taylor HR. Moraxella keratitis: predisposing factors and clinical review of 95 cases. Br J Ophthalmol 2006;90:1236-1238.
32. Yang KS, Lin HC, Ma DH, Chen HC, Tan HY, Huang SC, Lin KK, Hsiao CH. Ulcerative keratitis caused by Haemophilus influenzae. Cornea 2006;25:701-704.
33. Passos RM, Cariello AJ, Yu MC, Hofling-Lima AL. Microbial keratitis in the elderly: a 32-year review. Arq Bras Oftalmol 2010;73:315-319.
34. Green M, Apel A, Stapleton F. Risk factors and causative organisms in microbial keratitis. Cornea 2008;27:22-27.
35. Zhang C, Liang Y, Deng S, Wang Z, Li R, Sun X. Distribution of bacterial keratitis and emerging resistance to antibiotics in China from 2001 to 2004. Clinical ophthalmology (Auckland, N Z) 2008;2:575-579.
36. Alexandrakis G, Alfonso EC, Miller D. Shifting trends in bacterial keratitis in south Florida and emerging resistance to fluoroquinolones. Ophthalmology 2000;107:1497-1502.
37. Sueke H, Kaye S, Neal T, Murphy C, Hall A, Whittaker D, Tuft S, Parry C. Minimum inhibitory concentrations of standard and novel antimicrobials for isolates from bacterial keratitis. Invest Ophthalmol Vis Sci 2010;51:2519-2524.
38. Bourcier T, Thomas F, Borderie V, Chaumeil C, Laroche L. Bacterial keratitis: predisposing factors, clinical and microbiological review of 300 cases. Br J Ophthalmol 2003;87:834-838.
39. Bharathi MJ, Ramakrishnan R, Shivakumar C, Meenakshi R, Lionalraj D. Etiology and

antibacterial susceptibility pattern of community-acquired bacterial ocular infections in a tertiary eye care hospital in south India. Indian J Ophthalmol 2010;58:497-507.

40. Wong TY, Fong KS, Tan DT. Clinical and microbial spectrum of fungal keratitis in Singapore: a 5-year retrospective study. Int Ophthalmol 1997;21:127-130.
41. Leck AK, Thomas PA, Hagan M, Kaliamurthy J, Ackuaku E, John M, Newman MJ, Codjoe FS, Opintan JA, Kalavathy CM, Essuman V, Jesudasan CA, Johnson GJ. Aetiology of suppurative corneal ulcers in Ghana and south India, and epidemiology of fungal keratitis. Br J Ophthalmol 2002;86:1211-1215.
42. Jurkunas U, Behlau I, Colby K. Fungal keratitis: changing pathogens and risk factors. Cornea 2009;28:638-643.
43. Keay LJ, Gower EW, Iovieno A, Oechsler RA, Alfonso EC, Matoba A, Colby K, Tuli SS, Hammersmith K, Cavanagh D, Lee SM, Irvine J, Stulting RD, Mauger TF, Schein OD. Clinical and microbiological characteristics of fungal keratitis in the United States, 2001-2007: a multicenter study. Ophthalmology 2011;118:920-926.
44. Thomas PA, Leck AK, Myatt M. Characteristic clinical features as an aid to the diagnosis of suppurative keratitis caused by filamentous fungi. Br J Ophthalmol 2005;89:1554-1558.
45. Clarke B, Sinha A, Parmar DN, Sykakis E. Advances in the diagnosis and treatment of acanthamoeba keratitis. J Ophthalmol 2012;2012:484892.
46. Thiel MA, Bossart W, Bernauer W. Improved impression cytology techniques for the immunopathological diagnosis of superficial viral infections. Br J Ophthalmol 1997;81: 984-988.
47. Sawada Y, Yuan C, Huang AJ. Impression cytology in the diagnosis of acanthamoeba keratitis with surface involvement. Am J Ophthalmol 2004;137:323-328.
48. Kaufman SC, Musch DC, Belin MW, Cohen EJ, Meisler DM, Reinhart WJ, Udell IJ, Van Meter WS. Confocal microscopy: a report by the American Academy of Ophthalmology. Ophthalmology 2004;111:396-406.
49. El-Aal AM, El Sayed M, Mohammed E, Ahmed M, Fathy M. Evaluation of herpes simplex detection in corneal scrapings by three molecular methods. Curr Microbiol 2006;52:379-382.
50. Kumar M, Mishra NK, Shukla PK. Sensitive and rapid polymerase chain reaction based

diagnosis of mycotic keratitis through single stranded conformation polymorphism. Am J Ophthalmol 2005;140:851-857.

51. Ahn M, Yoon KC, Ryu SK, Cho NC, You IC. Clinical aspects and prognosis of mixed microbial (bacterial and fungal) keratitis. Cornea 2011;30:409-413.
52. Swartz MN. Use of antimicrobial agents and drug resistance. New Engl J Med 1997;337:491-492.
53. Lowy FD. Antimicrobial resistance: the example of Staphylococcus aureus. J Clin Invest 2003;111:1265-1273.
54. Fintelmann RE, Hoskins EN, Lietman TM, Keenan JD, Gaynor BD, Cevallos V, Acharya NR. Topical fluoroquinolone use as a risk factor for in vitro fluoroquinolone resistance in ocular cultures. Arch Ophthalmol-Chic 2011;129:399-402.
55. Asbell PA, Gadaria N. Crisis management antibiotic resistance in ocular infections. Cataract & Refractive Surgery Today 2013:62-64.
56. Garg P, Sharma S, Rao GN. Ciprofloxacin-resistant Pseudomonas keratitis. Ophthalmology 1999;106:1319-1323.
57. Moshirfar M, Meyer JJ, Espandar L. Fourth-generation fluoroquinolone-resistant mycobacterial keratitis after laser in situ keratomileusis. J Cataract Refr Surg 2007;33: 1978-1981.
58. Goldstein MH, Kowalski RP, Gordon YJ. Emerging fluoroquinolone resistance in bacterial keratitis: a 5-year review. Ophthalmology 1999;106:1313-1318.
59. Chuang CC, Hsiao CH, Tan HY, Ma DH, Lin KK, Chang CJ, Huang YC. Staphylococcus aureus ocular infection: methicillin-resistance, clinical features, and antibiotic susceptibilities. PLoS One 2012;8:e42437.
60. Haas W, Pillar CM, Torres M, Morris TW, Sahm DF. Monitoring antibiotic resistance in ocular microorganisms: results from the Antibiotic Resistance Monitoring in Ocular micRorganisms (ARMOR) 2009 surveillance study. Am J Ophthalmol 2011;152:567-574 e563.
61. Mah FS. Fourth-generation fluoroquinolones: new topical agents in the war on ocular bacterial infections. Curr Opin Ophthalmol 2004;15:316-320.

62. Wong RL, Gangwani RA, Yu LW, Lai JS. New treatments for bacterial keratitis. J Ophthalmol 2012;2012:831502.

63. Martins SA, Combs JC, Noguera G, Camacho W, Wittmann P, Walther R, Cano M, Dick J, Behrens A. Antimicrobial efficacy of riboflavin/UVA combination (365 nm) in vitro for bacterial and fungal isolates: a potential new treatment for infectious keratitis. Invest Ophthalmol Vis Sci 2008;49:3402-3408.

64. Schrier A, Greebel G, Attia H, Trokel S, Smith EF. In vitro antimicrobial efficacy of riboflavin and ultraviolet light on Staphylococcus aureus, methicillin-resistant Staphylococcus aureus, and Pseudomonas aeruginosa. J Refract Surg 2009;25:S799-802.

65. Raab O. Über die Wirkung fluoreszierender Stoffe auf Infusorien. Z Biol (Muench) 1900;39: 524-546.

66. Von Tappeiner H, Jesionek A. Therapeutische Versuche mit fluoreszierenden Stoffen. Muench Med Wochenschr 1903;47:2042-2044.

67. Pass HI. Photodynamic therapy in oncology: mechanisms and clinical use. J Natl Cancer Inst 1993;85:443-456.

68. Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem Photobiol Sci 2004;3:436-450.

69. Bozzini G, Colin P, Betrouni N, Nevoux P, Ouzzane A, Puech P, Villers A, Mordon S. Photodynamic therapy in urology: what can we do now and where are we heading? Photodiagnosis Photodyn Ther 2012;9:261-273.

70. Juarranz A, Jaen P, Sanz-Rodriguez F, Cuevas J, Gonzalez S. Photodynamic therapy of cancer. Basic principles and applications. Clin Transl Oncol 2008;10:148-154.

71. Moan J, Berg K. The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. Photochem Photobiol 1991;53:549-553.

72. Weishaupt KR, Gomer CJ, Dougherty TJ. Identification of singlet oxygen as the cytotoxic agent in photoinactivation of a murine tumor. Cancer Res 1976;36:2326-2329.

73. Henderson BW, Dougherty TJ. How does photodynamic therapy work? Photochem Photobiol 1992;55:145-157.

74. Rosenthal I, Ben-Hur E. Role of oxygen in the phototoxicity of phthalocyanines. Int J Radiat

Biol 1995;67:85-91.

75. Nowis D, Makowski M, Stoklosa T, Legat M, Issat T, Golab J. Direct tumor damage mechanisms of photodynamic therapy. Acta Biochim Pol 2005;52:339-352.
76. Ragas X, Agut M, Nonell S. Singlet oxygen in Escherichia coli: New insights for antimicrobial photodynamic therapy. Free Radic Biol Med 2010;49:770-776.
77. Huang L, Xuan Y, Koide Y, Zhiyentayev T, Tanaka M, Hamblin MR. Type I and Type II mechanisms of antimicrobial photodynamic therapy: an in vitro study on gram-negative and gram-positive bacteria. Lasers Surg Med 2012;44:490-499.
78. Ragas X, He X, Agut M, Roxo-Rosa M, Gonsalves AR, Serra AC, Nonell S. Singlet oxygen in antimicrobial photodynamic therapy: photosensitizer-dependent production and decay in E. coli. Molecules 2013;18:2712-2725.
79. Taylor HR, West S, Munoz B, Rosenthal FS, Bressler SB, Bressler NM. The long-term effects of visible light on the eye. Arch Ophthalmol 1992;110:99-104.
80. Ackroyd R, Kelty C, Brown N, Reed M. The history of photodetection and photodynamic therapy. Photochem Photobiol 2001;74:656-669.
81. De Jode ML, McGilligan JA, Dilkes MG, Cameron I, Hart PB, Grahn MF. A comparison of novel light sources for photodynamic therapy. Lasers Med Sci 1997;12:260-268.
82. Brancaleon L, Moseley H. Laser and non-laser light sources for photodynamic therapy. Lasers Med Sci 2002;17:173-186.
83. MacDonald IJ, Dougherty TJ. Basic principles of photodynamic therapy. J Porphyr Phthalocyanines 2001;5:105-129.
84. Wilson B, Patterson M, Burns D. Effect of photosensitizer concentration in tissue on the penetration depth of photoactivating light. Lasers Med Sci ; 1986;1:235-244
85. Lee LK, Whitehurst C, Pantelides ML, Moore JV. In situ comparison of 665 nm and 633 nm wavelength light penetration in the human prostate gland. Photochem Photobiol 1995;62: 882-886.
86. Allison RR, Downie GH, Cuenca R, Hu XH, Childs CJH, Sibata CH. Photosensitizers in clinical PDT. Photodiagnosis Photodyn Ther 2004;1:27-42.
87. Gomer CJ. Preclinical examination of first and second generation photosensitizers used in

photodynamic therapy. Photochem Photobiol 1991;54:1093-1107.

88. Josefsen LB, Boyle RW. Photodynamic therapy: novel third-generation photosensitizers one step closer? Br J Pharmacol 2008;154:1-3.
89. Dougherty TJ, Kaufman JE, Goldfarb A, Weishaupt KR, Boyle D, Mittleman A. Photoradiation therapy for the treatment of malignant tumors. Cancer Res 1978;38:2628-2635.
90. Roberts WG, Shiau FY, Nelson JS, Smith KM, Berns MW. In vitro characterization of monoaspartyl chlorin e6 and diaspartyl chlorin e6 for photodynamic therapy. J Natl Cancer Inst 1988;80:330-336.
91. Kostenich GA, Zhuravkin IN, Furmanchuk AV, Zhavrid EA. Sensitivity of different rat tumour strains to photodynamic treatment with chlorin e6. J Photochem Photobiol B 1993;17: 187-194.
92. Kostenich GA, Zhuravkin IN, Zhavrid EA. Experimental grounds for using chlorin e6 in the photodynamic therapy of malignant tumors. J Photochem Photobiol B 1994;22:211-217.
93. Moon YH, Kwon SM, Kim HJ, Jung KY, Park JH, Kim SA, Kim YC, Ahn SG, Yoon JH. Efficient preparation of highly pure chlorin e6 and its photodynamic anti-cancer activity in a rat tumor model. Oncol Rep 2009;22:1085-1091.
94. Cassell GH, Mekalanos J. Development of antimicrobial agents in the era of new and reemerging infectious diseases and increasing antibiotic resistance. JAMA 2001;285: 601-605.
95. Cerveny KE, DePaola A, Duckworth DH, Gulig PA. Phage therapy of local and systemic disease caused by Vibrio vulnificus in iron-dextran-treated mice. Infect Immun 2002;70: 6251-6262.
96. Park JH, Ahn MY, Kim YC, Kim SA, Moon YH, Ahn SG, Yoon JH. In vitro and in vivo antimicrobial effect of photodynamic therapy using a highly pure chlorin e6 against Staphylococcus aureus Xen29. Biol Pharm Bull 2012;35:509-514.
97. Szentmáry N, Goebels S, Bischoff M, Seitz B. Photodynamic therapy for infectious keratitis. Ophthalmologe 2012;109:165-170.
98. Wallis C, Melnick JL. Photodynamic inactivation of poliovirus. Virology 1963;21:332-341.

99. Wallis C, Melnick JL. Irreversible photosensitization of viruses. Virology 1964;23:520-527.

100. Wallis C, Melnick JL. Photodynamic inactivation of animal viruses: a review. Photochem Photobiol 1966;4:159-170.

101. Wallis C, Melnick JL. Photodynamic inactivation of enteroviruses. J Bacteriol 1965;89: 41-46.

102. Moore C, Wallis C, Melnick JL, Kuns MD. Photodynamic treatment of herpes keratitis. Infect Immun 1972;5:169-171.

103. Bartholomew RS, Clarke M, Phillips CI. "Dye/light" dye-induced photosensitization of herpes virus. A clinical trial on humans. Trans Ophthalmol Soc U K 1977;97:508-509.

104. Chen Z, Xuguang S, Zhiqun W, Ran L. In vitro amoebacidal activity of photodynamic therapy on Acanthamoeba. Br J Ophthalmol 2008;92:1283-1286.

105. Mito T, Suzuki T, Kobayashi T, Zheng X, Hayashi Y, Shiraishi A, Ohashi Y. Effect of photodynamic therapy with methylene blue on Acanthamoeba in vitro. Invest Ophthalmol Vis Sci 2012;53:6305-6313.

106. Ferro S, Coppellotti O, Roncucci G, Ben Amor T, Jori G. Photosensitized inactivation of Acanthamoeba palestinensis in the cystic stage. J Appl Microbiol 2006;101:206-212.

107. Del Buey MA, Cristobal JA, Casas P, Goni P, Clavel A, Minguez E, Lanchares E, Garcia A, Calvo B. Evaluation of in vitro efficacy of combined riboflavin and ultraviolet a for Acanthamoeba isolates. Am J Ophthalmol 2012;153:399-404.

108. Khan YA, Kashiwabuchi RT, Martins SA, Castro-Combs JM, Kalyani S, Stanley P, Flikier D, Behrens A. Riboflavin and ultraviolet light a therapy as an adjuvant treatment for medically refractive Acanthamoeba keratitis: report of 3 cases. Ophthalmology 2011;118:324-331.

109. Ito T. Photodynamic action of hematoporphyrin on yeast cells - a kinetic approach. Photochem Photobiol 1981;34:521-524.

110. Sauer A, Letscher-Bru V, Speeg-Schatz C, Touboul D, Colin J, Candolfi E, Bourcier T. In vitro efficacy of antifungal treatment using riboflavin/UV-A (365 nm) combination and amphotericin B. Invest Ophthalmol Vis Sci 2010;51:3950-3953.

111. Donnelly RF, McCarron PA, Tunney MM. Antifungal photodynamic therapy. Microbiol Res 2008;163:1-12.

112. Li Z, Jhanji V, Tao X, Yu H, Chen W, Mu G. Riboflavin/ultravoilet light-mediated crosslinking for fungal keratitis. Br J Ophthalmol 2013;97:669-671.

113. Huang L, Dai T, Hamblin MR. Antimicrobial photodynamic inactivation and photodynamic therapy for infections. Methods Mol Biol 2010;635:155-173.

114. Park JH, Moon YH, Bang IS, Kim YC, Kim SA, Ahn SG, Yoon JH. Antimicrobial effect of photodynamic therapy using a highly pure chlorin e6. Lasers Med Sci 2010;25:705-710.

115. Yow CM, Fung K, Wong KC. Photodynamic inactivation of multi-drug resistant pathogens in Hong Kong. Hong Kong Med J 2011;17 Suppl 2:24-28.

116. Thakuri PS, Joshi R, Basnet S, Pandey S, Taujale SD, Mishra N. Antibacterial photodynamic therapy on Staphylococcus aureus and Pseudomonas aeruginosa in-vitro. Nepal Med Coll J 2011;13:281-284.

117. Pollhammer M, Cursiefen C. Bacterial keratitis early after corneal crosslinking with riboflavin and ultraviolet-A. J Cataract Refract Surg 2009;35:588-589.

118. Price MO, Tenkman LR, Schrier A, Fairchild KM, Trokel SL, Price FW, Jr. Photoactivated riboflavin treatment of infectious keratitis using collagen cross-linking technology. J Refract Surg 2012;28:706-713.

119. Makdoumi K, Mortensen J, Sorkhabi O, Malmvall BE, Crafoord S. UVA-riboflavin photochemical therapy of bacterial keratitis: a pilot study. Graefes Arch Clin Exp Ophthalmol 2012;250:95-102.

120. Seitz B, Hayashi S, Wee WR, LaBree L, McDonnell PJ. In vitro effects of aminoglycosides and fluoroquinolones on keratocytes. Invest Ophthalmol Vis Sci 1996;37:656-665.

121. Cubitt CL, Tang Q, Monteiro CA, Lausch RN, Oakes JE. IL-8 gene expression in cultures of human corneal epithelial cells and keratocytes. Invest Ophthalmol Vis Sci 1993;34: 3199-3206.

122. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254.

123. Wollensak G, Sporl E, Seiler T. Treatment of keratoconus by collagen cross linking. Ophthalmologe 2003;100:44-49.

124. Wollensak G, Spoerl E, Reber F, Seiler T. Keratocyte cytotoxicity of riboflavin/UVA-

treatment in vitro. Eye (Lond) 2004;18:718-722.

125. Mencucci R, Marini M, Paladini I, Sarchielli E, Sgambati E, Menchini U, Vannelli GB. Effects of riboflavin/UVA corneal cross-linking on keratocytes and collagen fibres in human cornea. Clin Experiment Ophthalmol 2010;38:49-56.
126. Joseph A, Hossain P, Jham S, Jones RE, Tighe P, McIntosh RS, Dua HS. Expression of CD34 and L-selectin on human corneal keratocytes. Invest Ophthalmol Vis Sci 2003;44: 4689-4692.
127. Sosnová M, Bradl M, Forrester JV. CD34+ corneal stromal cells are bone marrow-derived and express hemopoietic stem cell markers. Stem Cells 2005;23:507-515.
128. Dua HS. Epithelial mesenchymal transition in the cornea. EuCornea Congress, Milan, Italy:2012.
129. Jester JV, Ho-Chang J. Modulation of cultured corneal keratocyte phenotype by growth factors/cytokines control in vitro contractility and extracellular matrix contraction. Exp Eye Res 2003;77:581-592.
130. Ebihara N, Yamagami S, Chen L, Tokura T, Iwatsu M, Ushio H, Murakami A. Expression and function of toll-like receptor-3 and -9 in human corneal myofibroblasts. Invest Ophthalmol Vis Sci 2007;48:3069-3076.
131. Espana EM, Kawakita T, Liu CY, Tseng SC. CD-34 expression by cultured human keratocytes is downregulated during myofibroblast differentiation induced by TGF-beta1. Invest Ophthalmol Vis Sci 2004;45:2985-2991.
132. Brissette-Storkus CS, Reynolds SM, Lepisto AJ, Hendricks RL. Identification of a novel macrophage population in the normal mouse corneal stroma. Invest Ophthalmol Vis Sci 2002;43:2264-2271.
133. Hamrah P, Huq SO, Liu Y, Zhang Q, Dana MR. Corneal immunity is mediated by heterogeneous population of antigen-presenting cells. J Leukoc Biol 2003;74:172-178.
134. Hu MC, Chien SL. The cytoplasmic domain of stem cell antigen CD34 is essential for cytoadhesion signaling but not sufficient for proliferation signaling. Blood 1998;91: 1152-1162.
135. Usuda J, Okunaka T, Furukawa K, Tsuchida T, Kuroiwa Y, Ohe Y, Saijo N, Nishio K,

Konaka C, Kato H. Increased cytotoxic effects of photodynamic therapy in IL-6 gene transfected cells via enhanced apoptosis. Int J Cancer 2001;93:475-480.

136. Okunaka T, Usuda J, Ichinose S, Hirata H, Ohtani K, Maehara S, Inoue T, Imai K, Kubota M, Tsunoda Y, Kuroiwa Y, Tsutsui H, Furukawa K, Nishio K, Kato H. A possible relationship between the anti-cancer potency of photodynamic therapy using the novel photosensitizer ATX-s10-Na(II) and expression of the vascular endothelial growth factor in vivo. Oncology reports 2007;18:679-683.

137. Wilson SE, Lloyd SA, Kennedy RH. Epidermal growth factor messenger RNA production in human lacrimal gland. Cornea 1991;10:519-524.

138. Pasquale LR, Dorman-Pease ME, Lutty GA, Quigley HA, Jampel HD. Immunolocalization of TGF-beta 1, TGF-beta 2, and TGF-beta 3 in the anterior segment of the human eye. Invest Ophthalmol Vis Sci 1993;34:23-30.

139. Wilson SE, Chen L, Mohan RR, Liang Q, Liu J. Expression of HGF, KGF, EGF and receptor messenger RNAs following corneal epithelial wounding. Exp Eye Res 1999;68:377-397.

140. Ley RD, Miska KB, Kusewitt DF. Photoreactivation of ultraviolet radiation-induced basic fibroblast growth factor (bFGF) and the role of bFGF in corneal lesion formation in Monodelphis domestica. Environ Mol Mutagen 2001;38:175-179.

141. Carrington LM, Boulton M. Hepatocyte growth factor and keratinocyte growth factor regulation of epithelial and stromal corneal wound healing. J Cataract Refract Surg 2005;31: 412-423.

142. Lee YH, Marquez AP, Mungunsukh O, Day RM. Hepatocyte growth factor inhibits apoptosis by the profibrotic factor angiotensin II via extracellular signal-regulated kinase 1/2 in endothelial cells and tissue explants. Mol Biol Cell 2010;21:4240-4250.

143. Wilson SE, Schultz GS, Chegini N, Weng J, He YG. Epidermal growth factor, transforming growth factor alpha, transforming growth factor beta, acidic fibroblast growth factor, basic fibroblast growth factor, and interleukin-1 proteins in the cornea. Exp Eye Res 1994;59: 63-71.

144. Hayashi M, Hayashi Y, Liu CY, Tichelaar JW, Kao WW. Over expression of FGF7 enhances cell proliferation but fails to cause pathology in corneal epithelium of Kerapr-rtTA/FGF7

bitransgenic mice. Mol Vis 2005;11:201-207.

145. Sotozono C, Inatomi T, Nakamura M, Kinoshita S. Keratinocyte growth factor accelerates corneal epithelial wound healing in vivo. Invest Ophthalmol Vis Sci 1995;36:1524-1529.

146. Bock F, Onderka J, Dietrich T, Bachmann B, Kruse FE, Paschke M, Zahn G, Cursiefen C. Bevacizumab as a potent inhibitor of inflammatory corneal angiogenesis and lymphangiogenesis. Invest Ophthalmol Vis Sci 2007;48:2545-2552.

147. West-Mays JA, Dwivedi DJ. The keratocyte: corneal stromal cell with variable repair phenotypes. Int J Biochem Cell Biol 2006;38:1625-1631.

148. Honma Y, Nishida K, Sotozono C, Kinoshita S. Effect of transforming growth factor-beta1 and -beta2 on in vitro rabbit corneal epithelial cell proliferation promoted by epidermal growth factor, keratinocyte growth factor, or hepatocyte growth factor. Exp Eye Res 1997;65: 391-396.

149. Wang J, Stachon T, Eppig T, Langenbucher A, Seitz B, Szentmáry N. Impact of photodynamic inactivation (PDI) on viability, apoptosis and proliferation of human keratocytes in vitro. Invest Ophthalmol Vis Sci 2012;53:E-Abstract 1092.

150. Szentmáry N, Stachon T, Wang J, Eppig T, Langenbucher A, Seitz B. CD34 und alpha-smooth muscle actin Keratozyten Expression nach photodynamischer Inaktivierung (PDI). Klin Monatsbl Augenheilkd 2013:In press.

151. Chu R, Zheng X, Chen D, Hu DN. Blue light irradiation inhibits the production of HGF by human retinal pigment epithelium cells in vitro. Photochem Photobiol 2006;82:1247-1250.

152. Wollensak G, Mazzotta C, Kalinski T, Sel S. Limbal and conjunctival epithelium after corneal cross-linking using riboflavin and UVA. Cornea 2011;30:1448-1454.

153. Cho KS, Lee EH, Choi JS, Joo CK. Reactive oxygen species-induced apoptosis and necrosis in bovine corneal endothelial cells. Invest Ophthalmol Vis Sci 1999;40:911-919.

154. Hovakimyan M, Guthoff RF, Stachs O. Collagen cross-linking: current status and future directions. J Ophthalmol 2012;2012:406850.

155. Saczko J, Mazurkiewicz M, Chwilkowska A, Kulbacka J, Kramer G, Lugowski M, Snietura M, Banas T. Intracellular distribution of Photofrin in malignant and normal endothelial cell lines. Folia Biol (Praha) 2007;53:7-12.

156. Wawrzynska M, Kalas W, Bialy D, Ziolo E, Arkowski J, Mazurek W, Strzadala L. In vitro photodynamic therapy with chlorin e6 leads to apoptosis of human vascular smooth muscle cells. Arch Immunol Ther Exp (Warsz) 2010;58:67-75.

157. Simon C, Wolf G, Hüttenberger D, Foth H, Seitz B. Penetration of the photosensitizer chlorin e6 into the cornea for photodynamic inactivation in infectious keratitis. 109th DOG-Congress, Berlin, Germany 2011.

158. Valtink M, Gruschwitz R, Funk RH, Engelmann K. Two clonal cell lines of immortalized human corneal endothelial cells show either differentiated or precursor cell characteristics. Cells Tissues Organs 2008;187:286-294.

159. Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. Am J Ophthalmol 2003;135:620-627.

160. Wollensak G, Sporl E, Reber F, Pillunat L, Funk R. Corneal endothelial cytotoxicity of riboflavin/UVA treatment in vitro. Ophthalmic Res 2003;35:324-328.

161. Wollensak G, Spoerl E, Wilsch M, Seiler T. Endothelial cell damage after riboflavin-ultraviolet-A treatment in the rabbit. J Cataract Refract Surg 2003;29:1786-1790.

162. Micelli Ferrari T, Leozappa M, Lorusso M, Epifani E, Micelli Ferrari L. Escherichia coli keratitis treated with ultraviolet A/riboflavin corneal cross-linking: a case report. Eur J Ophthalmol 2009;19:295-297.

163. Embleton ML, Nair SP, Cookson BD, Wilson M. Antibody-directed photodynamic therapy of methicillin resistant Staphylococcus aureus. Microb Drug Resist 2004;10:92-97.

164. Demidova TN, Hamblin MR. Photodynamic therapy targeted to pathogens. Int J Immunopathol Pharmacol 2004;17:245-254.

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PUBLICATIONS AND CONFERENCE PARTICIPATIONS

Scientific publications

1. Wang J, Hasenfus A, Schirra F, Bohle RM, Seitz B, Szentmáry N. Changing indications for penetrating keratoplasty in Homburg/Saar from 2001 to 2010-histopathology of 1,200 corneal buttons. Graefes Arch Clin Exp Ophthalmol 2013; 251(3):797-802. **(IF: 2.17)**

2. Wang J, Stachon T, Eppig T, Langenbucher A, Seitz B, Szentmáry N. Impact of photodynamic inactivation (PDI) using the photosensitizer chlorin e6 on viability, apoptosis, and proliferation of human corneal endothelial cells. Graefes Arch Clin Exp Ophthalmol 2013; 251(4):1199-1204. **(IF: 2.17)**

3. Stachon T, Wang J, Eppig T, Langenbucher A, Foth H-J, Bischoff M, Seitz B, Szentmáry N. KGF, FGFb, VEGF, HGF and TGFβ1 secretion of human keratocytes following photodynamic inactivation (PDI) in vitro. Graefes Arch Clin Exp Ophthalmol 2013; [*Epub ahead of print*]. **(IF: 2.17)**

4. Szentmáry N, Wang J, Stachon T, Goebels S, Seitz B. CD34 und alpha-smooth muscle actin Keratozyten Expression nach photodynamischer Inaktivierung (PDI). Klin Monatsbl Augenheilkd 2013; *in press*. **(IF: 0.51)**

5. Wang J, Stachon T, Eppig T, Langenbucher A, Seitz B, Szentmáry N. Impact of photodynamic inactivation (PDI) using the photosensitizer chlorin e6 on viability, apoptosis, and proliferation of human keratocytes in vitro. Graefes Arch Clin Exp Ophthalmol 2013; *Submitted.*

Scientific talks

1. Wang J, Hasenfus A, Schirra F, Bohle RM, Seitz B, Szentmáry N. Changing indications for penetrating keratoplasty in Homburg/Saar from 2001 to 2010-histopathology of 1,200 corneal buttons. 109. DOG Kongress, Berlin, 01.10.2011.

2. Wang J, Hasenfus A, Schirra F, Bohle RM, Seitz B, Szentmáry N. Changing indications for penetrating keratoplasty in Homburg/Saar from 2001 to 2010-histopathology of 1,200 corneal buttons. 84. Versammlung der Rhein-Mainischen Augenärzte, Marburg, 05.11. 2011.

3. Szentmáry N, Wang J, Stachon T, Eppig T, Langenbucher A, Foth HJ, Seitz B. Apoptosis, proliferation and viability of human corneal keratocyte cell cultures following photodynamic therapy. 84. Versammlung der Rhein-Mainischen Augenärzte, Marburg, 05.11.2011. **(1. Preis)**

Scientific presentations

1. Wang J, Stachon T, Seitz B, Eppig T, Langenbucher A, Foth HJ, Szentmáry N. Impact of photodynamic therapy on the viability of cultured human corneal keratocytes. Poster, 109. DOG Kongress, Berlin, 29.09. – 02.10.2011.

2. Wang J, Stachon T, Bischoff M, Foth H-J, Eppig T, Langenbucher A, Seitz B, Szentmáry N. Impact of photodynamic therapy (PDT) on viability, apoptosis and proliferation of human keratocytes in vitro. Invest Ophthalmol Vis Sci 2012; 53: E-Abstract 1092.

3. Wang J, Stachon T, Eppig T, Langenbucher A, Seitz B, Szentmáry N. Impact of photodynamic inactivation (PDI) on viability, apoptosis and proliferation of human corneal endothelial cells. Poster, 110. DOG Kongress, Berlin, 20.09. – 23.09.2012.

4. Stachon T, Wang J, Eppig T, Langenbucher A, Foth H-J, Seitz B, Szentmáry N. Apoptosis and proliferation induced by photodynamic therapy in human keratocyte cell cultures. Poster, 109. DOG Kongress, Berlin, 29.09. – 02.10.2011.

5. Stachon T, Wang J, Eppig T, Langenbucher A, Seitz B, Szentmáry N. Apoptosis and viability of human corneal endothelial cell cultures following photodynamic therapy (PDT). Invest Ophthalmol Vis Sci 2012; 53: E-Abstract 6014.

6. Stachon T, Wang J, Eppig T, Langenbucher A, Bischoff M, Foth H-J, Seitz B, Szentmáry N. Impact of photodynamic therapy (PDT) on bFGF, HGF, KGF, TGFβ1 and VEGF secretion of keratocytes in vitro. Poster, 110. DOG Kongress, Berlin, 20.09. – 23.09.2012. **(1. Poster preis)**

7. Szentmáry N, Stachon T, Wang J, Eppig T, Langenbucher A, Bischoff M, Foth H-J, Seitz B. Photodynamic therapy triggers expression of haemopoietic stem cell marker CD34 of keratocytes. Invest Ophthalmol Vis Sci 2012; 53: E-Abstract 1078.

8. Winkler K, Finke M, Wang J, Szentmáry N, Eppig T, Foth H-J, Hüttenberger D, Langenbucher A, Seitz B, Bischoff M. In vitro effectiveness of photodynamic therapy against multi-resistant pathogens. Invest Ophthalmol Vis Sci 2012; 53: E-Abstract 6206.

9. Song XF, Gkatzioufas Z, Wang J, Stachon T, Seitz B. Effect of thyroxine and hydrocortisone on human keratocyte viability and proliferation in vitro. Poster, 110. DOG Kongress, Berlin, 20.09. – 23.09.2012.

10. Szentmáry N, Stachon T, Wang J, Eppig T, Langenbucher A, Bischoff M, Foth HJ, Seitz B. CD34 and alpha-smooth actin expression of keratocytes following photodynamic inactivation (PDI). Poster, Congress of the Hungarian Ophthalmological Society, Siófok, Hungary, 2012.

11. Szentmáry N, Stachon T, Wang J, Eppig T, Langenbucher A, Seitz B. CD34 and alpha-smooth actin expression of keratocytes following photodynamic inactivation (PDI). Poster, Eucornea Kongress, Mailand, Italien, 2012.

12. Szentmáry N, Stachon T, Wang J, Eppig T, Langenbucher A, Bischoff M, Foth HJ, Seitz B. Impact of photodynamic inactivation (PDI) on bFGF, HGF, KGF, TGFß1 and VEGF secretion of keratocytes in vitro. Poster, EVER Congress, Nice, France, 2012.

13. Finke M, Bleses K, Winkler K, Wang J, Szentmáry N, Eppig T, Foth H-J, Hüttenberger D, Langenbucher A, Seitz B, Bischoff M. Die photodynamische Therapie: eine neue Perspektive für die Behandlung der bakteriell induzierten infektiösen Keratitis. Ophthalmologe 2012; 109 (Suppl. 1):59 abstract PDo06-046.

14. Seitz B. Stachon T, Wang J, Langenbucher A, Szentmáry N. Secretion of IL-1α, IL-1β, IL-6 and IL-8 in human keratocyte cell cultures following photodynamic inactivation (PDI). Poster, ARVO Annual Meeting, Seattle, 05.05. – 09.05.2012

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