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Impact of Hormone Treatment and Photodynamic Therapy (PDT) on Human Keratoconus Keratocytes in Vitro

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Auswirkungen der Hormonbehandlung und Photodynamischen Therapie (PDT) auf humane Keratokonus Keratozyten in vitro

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1. Berichterstatter: PD Dr. Z. Gatzioufas, Ph.D.
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2. Berichterstatter:

3. Berichterstatter:

To

My Parents

&

Weishi Dong

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, 05.08.2014

Xuefei Song

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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
Cluster of differentiation 34	CD34
Coagulase-negative staphylococcus	CNS
Corneal collagen crosslinking	CXL
Drug-resistant streptococcus pneumoniae	DRSP
Dulbecco's modified eagle medium: nutrient mixture F-12	DMEM/F12
Ethylenediaminetetra-acetic acid	EDTA
Epithelium growth factor	EGF
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
Interleukin-1ß	IL-1ß
Interleukin-6	IL-6
Interleukin-8	IL-8
Infrared rays	IR
Keratinocyte growth factor	KGF
Keratoconus	KC
Macrophage inflammatory protein-1α	MIP-1α
Multidrug-resistant pseudomonas aeruginosa	MDRPA
Matrix metalloproteinase-9	MMP-9
Methicillin-resistant staphylococcus aureus	MRSA

One-way analysis of variance	ANOVA
Penicillin/streptomycin	P/S
Phosphate-buffered saline	PBS
Photodynamic inactivation	PDI
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 Hormone Treatment and Photodynamic Therapy (PDT) on Human Keratoconus Keratocytes in Vitro

Background and purposes: Keratoconus is a multi-factorial disease causing corneal degeneration, thinning and secondary ectasia, which can severely degrade visual performance. Its pathophysiology is unknown but suggested aetiological factors include oxidative damage, repetitive mechanical injury, immunological factors and genetic factors. In addition, hormonal factors have been shown to play a potential role. 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 keratoconus keratocytes.

The *purpose* of this study was:

- To investigate the impact of thyroxine and hydrocortisone on the viability, proliferation and apoptosis of human keratocytes *in vitro* in normal and keratoconus corneas.
- To investigate the impact of thyroxine and hydrocortisone on the secretion of growth factors and interleukins by normal human keratocytes and keratoconus keratocytes *in vitro*.
- To determine the impact of riboflavin-UVA-PDT on viability, apoptosis, proliferation and activation of human keratoconus keratocytes, *in vitro.*
- To determine the impact of crosslinking on growth factor and interleukin secretion of human keratoconus keratocytes, 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. Keratocytes either were seeded and treated with thyroxine and hydrocortisone in concentrations of 0, 1, 10 and 20 µg/ml for 24

hours, or underwent UVA illumination (370 nm) for 4 minutes and 10 seconds with an irradiation dose of 8 mW/cm^2 (2 J/cm^2) during exposure to 0.05% or 0.1% riboflavin and 20% dextran in PBS. In supplementary experiment of cell proliferation test, cells were also treated with Fibroblast Growth Factor basic (FGFb) in 1ng/ml, and Transforming Growth Factor beta 1 (TGFβ1) in 2ng/ml for 24h.

Twenty-four hours after hormone treatment or PDT, human keratocyte viability was evaluated by the Alamar blue assay, apoptosis and total DNA content of the cells using the APO-DIRECT ™ Kit and cell proliferation by the BrdU Cell Proliferation Assay Kit.

CD34 and alpha smooth muscle actin (α-SMA) expression of human keratocytes was analyzed using flow cytometry (FACS) after illumination following exposure to 0.1% riboflavin in the culture medium. In addition, five and twenty-four hours after hormone treatment or illumination, the secretion of IL-1β, IL-6, IL-8, FGFb, HGF, KGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα of human keratocytes was measured by enzyme-linked immunoabsorbent assay (ELISA).

Results: Twenty-four hours after hormone treatment, we did not observe any changes in cell viability or apoptosis of normal keratocytes and keratoconus keratocytes. Cell proliferation of normal keratocytes increased significantly 24h after treatment with thyroxine from concentration of 1 μg/ml and higher ($p<0.01$), but there were no changes with hydrocortisone. Significant increase of proliferation in keratoconus keratocytes was only detected in the 20 μg/ml thyroxine group ($p<0.05$), and there were also no changes with hydrocortisone. Proliferation increased on normal keratocytes ($p<0.01$, $p<0.01$) and decreased on keratoconus keratocytes ($p<0.01$, $p<0.01$) significantly after FGFb and TGFβ1 treatment for 24h.

In normal keratocytes, treatment with thyroxine significantly down-regulated the secretion of IL-6 at 5h and up-regulated the secretion of HGF at 24h, while treatment with hydrocortisone significantly down-regulated the secretion of IL-6, IL-8 and FGFb at 5h, as well as the secretion of IL-6, IL-8, EGF and TGFβ1 at 24h. In keratoconus keratocytes, treatment with thyroxine significantly up-regulated the secretion of IL-8 and TNFa at 5h, as well as the secretion of HGF at 24h, whereas treatment with hydrocortisone significantly down-regulated the secretion of IL-6, IL-8 and TNFa at 5h, significantly down-regulated the secretion of IL-6 and TGFβ1 at 24h and significantly up-regulated the secretion of HGF at 24h.

Using riboflavin or UVA light only, we did not detect significant changes of cell viability, apoptosis, proliferation, CD34 or α-SMA expression. Using riboflavin-UVA-PDT, cell viability decreased significantly at 0.1% ($p<0.05$), the percentage of apoptotic KC keratocytes increased significantly at 0.05% and 0.1% ($p<0.05$ for both) and proliferation was inhibited at 0.05 and 0.1% ($p=0.009$ for both) riboflavin concentrations, compared to untreated controls. Following riboflavin-UVA-PDT, CD34 and alpha-smooth muscle actin expression of KC keratocytes remained unchanged compared to controls ($p>0.06$).

KGF and IL-1β secretion of keratocytes were below the measurement limit for all timepoints. Using riboflavin or UVA light illumination separately, growth factor and interleukin secretion of keratocytes remained unchanged for both time points ($p>0.35$). Five hours after crosslinking, FGFb secretion of keratoconus keratocytes increased ($p = 0.037$) significantly compared to untreated controls, whereas HGF, TGFβ1, VEGF, IL-6, and IL-8 secretion remained unchanged. 24 hours following CXL, none of the growth factor and interleukin concentrations differed significantly from untreated controls ($p>0.12$).

Conclusions:

- Thyroxine and hydrocortisone appear to have no significant impact on cell viability and apoptosis of human keratocytes *in vitro*. Treatment with thyroxine triggers the proliferation of normal keratocytes much more effectively than that of keratoconus keratocytes. FGFb and TGFβ1 increased proliferation of normal keratocytes and decreased that of keratoconus keratocytes. Hydrocortisone does not affect significantly cell proliferation of normal keratocytes and keratoconus keratocytes.
- Thyroxine significantly up-regulates the secretion of certain growth factors and interleukins by keratoconus keratocytes *in vitro*, although it does not affect their secretion by normal keratocytes. Hydrocortisone predominatly down-regulates the secretion of certain growth factors and interleukins by normal and keratoconus keratocytes. It appears that hormonal influences have differential effects on the secretion of these factors by human keratocytes, thereby modulating to some extent the corneal homeostasis.
- Crosslinking/riboflavin-UVA-PDT decreases viability, triggers apoptosis and inhibits proliferation, however this does not have an impact on multipotent

haematopoietic stem cell transformation and myofibroblastic transformation of human KC keratocytes *in vitro*.

- Crosslinking triggers FGFb secretion of *keratoconus* keratocytes transiently (5 hours), which normalizes after 24 hours. Crosslinking does not seem to have an impact on HGF, TGFβ1, VEGF, KGF, IL-1β, IL-6, and IL-8 secretion of keratoconus keratocytes in the short term.

ZUSAMMENFASSUNG

Auswirkung der Hormonbehandlung und der Photodynamischen Therapie (PDT) auf Humane Keratokonus Keratozyten *in Vitro*

Hintergrund/Ziele: Keratokonus stellt eine multifaktorielle Hornhautdegeneration dar, welche eine ausgeprägte Hornhautverdünnung und Hornhautektasie induziert, und dadurch die Sehschärfe erheblich beeinträchtigen kann. Die Pathophysiologie des Keratokonus ist nicht vollständig bekannt. Es wird allerdings vermutet, dass oxidativer Stress, mechanische Hornhautverletzung aufgrund regelmäßigen Augenreibens, immunologische Faktoren sowie genetische Faktoren eine wichtige Rolle spielen. Außerdem sind hormonelle Einflüsse von besonderer Bedeutung in der Pathogenese des Keratokonus. Die photodynamische Therapie (PDT) basiert auf der Photoaktivierung eines Photosensibilisators unter dem Einfluss von Licht einer bestimmten Wellenlänge, welches die Bildung von aggressiven freien Radikalen verursacht und dadurch zum Zelltod (zytotoxischer Effekt) führen kann. Aus diesen Gründen ist es sehr bedeutsam, die Auswirkung der PDT auf die humanen keratokonischen Keratozyten zu untersuchen.

Die Ziele dieser Studie sind:

- Die Untersuchung des Effekts von Thyroxin und Hydrokortison auf die Viabilität, Proliferation und Apoptose humaner Keratozyten aus normalen und keratokonischen Hornhäuten *in vitro*.
- Die Untersuchung des Effekts von Thyroxin und Hydrokortison auf die Sekretion verschiedener Wachstumsfaktoren und Interleukinen von normalen humanen Keratozyten und keratokonischen Keratozyten *in vitro.*
- Die Untersuchung des Effekts von Thyroxin und Hydrokortison auf die Expression von CD34 und alpha smooth muscle actin (α-SMA) von normalen humanen Keratozyten und keratokonischen Keratozyten *in vitro.*
- Die Untersuchung des Effekts von Riboflavin-UVA-PDT auf die Viabilität,

Proliferation und Apoptose humaner keratokonischer Keratozyten *in vitro*.

- Die Untersuchung des Effekts von Riboflavin-UVA-PDT auf die Sekretion verschiedener Wachstumsfaktoren und Interleukinen von humanen keratokonischen Keratozyten *in vitro.*
- Die Untersuchung des Effekts von Riboflavin-UVA-PDT auf die Expression von CD34 und alpha smooth muscle actin (α-SMA) von normalen humanen Keratozyten und keratokonischen Keratozyten *in vitro.*

Methode: Primäre humane Keratozyten wurden durch Kollagenasen-Verdau (1.0 mg/ml) aus humanen Hornhauttransplantaten isoliert und in DMEM/Ham's F12 Medium mit 10% fetalem Kälberserum kultiviert. Die Keratozyten wurden entweder mit Thyroxin und Hydrokortison in verschiedenen Konzentrationen (0, 1, 10 und 20 µg/ml) über 24 Stunden kultiviert, oder mit Riboflavin-UVA über einem Zeitraum von 4 Minuten und 10 Sekunden behandelt. Die Lichtquelle hat eine Wellenlänge von 375 nm und eine Bestrahlungsstärke von 8 mW/cm^2 bei einer Energiedosis von 2 J/cm^2. Riboflavin-5-phosphat wurde in Konzentrationen von 0%, 0.05% und 0.1% mit 20% Dextran in PBS eingesetzt. In weiteren Experimenten wurden die Keratozyten mit Fibroblast Growth Factor basic (FGFb) mit der Konzentration von 1 ng/ml, und Transforming Growth Factor beta 1 (TGFβ1) mit der Konzentration von 2 ng/ml über 24 Stunden kultiviert.

Vierundzwanzig Stunden nach Hormonbehandlung oder PDT wurde die Viabilität photometrisch mit dem alamarBlue® Assay, die Apoptose durchflusszytometrisch mit dem APO- DIRECT™ Kit und die Zellproliferation photometrisch mit dem BrdU Cell Proliferation ELISA Kit evaluiert.

Die Sekretion von IL-1β, IL-6, IL-8, FGFb, HGF, KGF, TGFβ1, EGF, MIP1α, MMP-9 und TNFα von humanen Keratozyten (5 Stunden und 24 Stunden) nach Hormonbehandlung oder Riboflavin-UVA-Bestrahlung wurde photometrisch mit einem Enzyme-linked immunosorbent assay (ELISA) bestimmt.

Die Bestimmung der Expression von CD34 und Alpha Smooth Muscle actin(α-SMA) erfolgte durchflusszytometrisch unter Verwendung spezifischer fluoreszenzmarkierter Antikörper.

Ergebnisse: Vierundzwanzig Stunden nach Hormonbehandlung konnten keine

Unterschiede der Viabilität und Apoptose zwischen normalen Keratozyten und keratokonischen Keratozyten nachgewiesen werden. Die Proliferation der normalen Keratozyten war signifikant erhöht nach der Applikation von Thyroxin ab der Konzentration von 1 µg/ml ($p<0.01$), aber es zeigten sich keine Unterschiede nach Hydrokortisongabe.

Die Proliferation der keratokonischen Keratozyten war nur signifikant erhöht mit einer Thyroxinkonzentration von 20 µg/ml ($p<0.05$) und es zeigten sich ebenfalls keine Unterschiede nach Hydrokortisongabe.

Nach Zugabe von FGFb und TGFβ1 über 24 Stunden stieg die Proliferation der normalen Keratozyten signifikant an ($p<0.01$, $p<0.01$) und die Proliferation der keratokonischen Keratozyten sank signifikant ab ($p<0.01$, $p<0.01$).

Die Applikation von Thyroxin zu normalen Keratozyten führte zur signifikanten Reduzierung der IL-6-Sekretion nach 5 Stunden und zu einem Anstieg der HGF-Sekretion nach 24 Stunden. Der Zusatz von Hydrokortison induzierte eine signifikante Abnahme der IL-6-, IL-8- und FGFb-Sekretion nach 5 Stunden, sowie der IL-6-, IL-8-, EGF- und TGFβ-Sekretion nach 24 Stunden. Die Zugabe von Thyroxin zu keratokonischen Keratozyten führte zu einem signifikanten Anstieg der IL-8- und TNFa-Expression nach 5 Stunden, sowie des Anstiegs der HGF-Sekretion nach 24 Stunden. Der Zusatz von Hydrokortison induzierte eine signifikante Abnahme der IL-6-, IL-8- und TNFa-Sekretion nach 5 Stunden, und der IL-6- und TGFβ-Sekretion nach 24 Stunden, sowie einen signifikanten Anstieg der HGF-Konzentration nach 24 Stunden.

Nach Riboflavin-Behandlung oder UVA-Bestrahlung konnten keine Unterschiede der Viabilität, Apoptose, Proliferation, CD34- oder α-SMA-Expression nachgewiesen werden. Nach Riboflavin-UVA-PDT sank die Viabilität signifikant bei der Konzentration von 0.1 % ($p<0.05$). Die Apoptose der keratokonischen Keratozyten stieg signifikant bei Konzentrationen von 0.05% und 0.1% ($p<0.05$). Die Proliferationsrate nahm bei Konzentrationen von 0.05 und 0.1% ($p=0.009$ für beide) im Vergleich zur Kontrolle ab. Nach Riboflavin-UVA-PDT blieb die Expression von CD34 und α-SMA der keratokonischen Keratozyten unverändert ($p>0.06$).

Nach Riboflavin-Behandlung oder UVA-Bestrahlung blieb die Sekretion von Wachstumsfaktoren und Interleukinen der Keratozyten unverändert ($p>0.35$). Fünf Stunden nach Crosslinking stieg die Sekretion von FGFb signifikant an ($p = 0.037$), wobei die Sekretion von HGF, TGFβ1, VEGF, IL-6, und IL-8 unverändert blieb.

Vierundzwanzig Stunden nach Crosslinking blieb die Expression von Wachstumsfaktoren und Interleukinen der Keratozyten unverändert ($p>0.12$).

Schlußvolgerungen:

- Thyroxin und Hydrokortison haben keinen Einfluss auf die Viabilität und Apoptose humaner Keratozyten *in vitro*. Die Zugabe von Thyroxin stimuliert die Proliferation normaler Keratozyten stärker als die Proliferation keratokonischer Keratozyten. FGFb und TGFβ1 induzieren einen Anstieg der Proliferation normaler Keratozyten und eine Abnahme der Proliferation keratokonischer Keratozyten. Hydrokortison hat keinen signifikanten Einfluss auf die Proliferation normaler und keratokonischer Keratozyten.
- Thyroxin stimuliert die Sekretion Wachstumsfaktoren und Interleukinen von keratokonischen Keratozyten *in vitro*, aber nicht die Sekretion von Wachstumsfaktoren und Interleukinen von normalen Keratozyten. Hydrokortison vermindert die Sekretion Wachstumsfaktoren und Interleukinen sowohl von normalen als auch von keratokonischen Keratozyten *in vitro*. Diese Ergebnisse weisen darauf hin, dass die hormonellen Einflüsse differenzierte Effekte auf die Sekretion von Wachstumsfaktoren und Interleukinen von normalen und keratotokonischen Keratozyten haben.
- Riboflavin-UVA-PDT reduziert die Viabilität und Proliferation und erhöht die Apoptose der Keratozyten. Allerdings zeigt sich keine Auswirkung auf die Stammzelltransformation und myofibroblastische Transformation humaner keratokonischer Keratozyten *in vitro*.
- Riboflavin-UVA-PDT induziert die Sekretion des FGFb von keratokonischen Keratozyten nach 5 Stunden, welche sich nach 24 Stunden normalisiert. Riboflavin-UVA-PDT hat keinen signifikanten Einfluss auf die Sekretion der HGF-, TGFβ1-, VEGF-, KGF-, IL-1β-, IL-6-, und IL-8-Expression von keratokonischen Keratozyten.

1 INTRODUCTION

Eye is the most sophisticated optical instrument in human body. A healthy, clear cornea is essential for good vision. Cornea 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.

Keratoconus is a bilateral corneal degeneration, which is characterized by marked corneal thinning and conical protrusion of the cornea, which can severely degrade visual performance.[2] The etiology and pathophysiology of keratoconus is multifactorial and has not been elucidated yet. A number of investigators, including our group, have reported that fluctuation of hormones and cytokines may play a function in the exacerbation or even initiation of keratoconus. [3-8]

In recent years, the technique of corneal collagen crosslinking (CXL) has been developed with its several advantages to stop the progression of keratoconus. [9,10] CXL is also being applied as an alternative treatment for persistent infectious keratitis and it offers a potential treatment alternative. [11,12]

In this work, we describe the challenges in diagnosis and management of keratoconus. We also investigate the impact of hormone treatment and PDT on human keratoconus keratocytes in vitro, which may play important roles in initiation, exacerbation or inhibition of progressive keratoconus.

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 adjacent epithelial cells provide a protective barrier, which is essential for good corneal optical properties during continuous renewal of the corneal epithelium. [13,14]

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. [15-17] The deficiency of limbal stem cells may result in conjunctival epithelial ingrowth, neovascularization, chronic inflammation or recurrent epithelial erosions and defects. [18,19]

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. [20]

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. [21,22]

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. [23]

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 adult zone; a posterior zone, which serves as a modified basement membrane for the endothelium. [24]

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. [25-27]

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, [28] diseases [29] and intraocular surgery. [30]

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

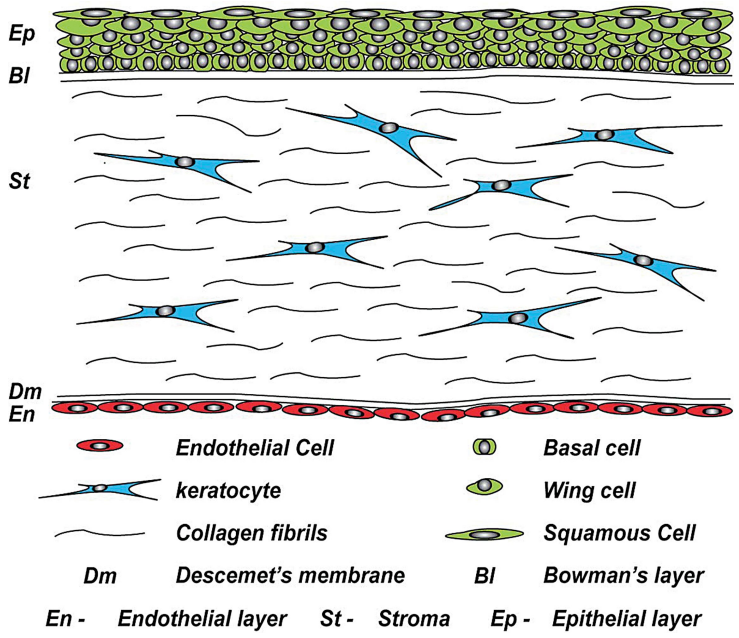

Figure 1. The human cornea in cross-section. [14]

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 Keratoconus

1.2.1 Definition and Epidemiology

Keratoconus (KC) is the most common primary ectasia, characterized by localized corneal thinning leading to protrusion of the thinned cornea. It usually occurs in the second decade of life and affects both genders and all ethnicities. [2,31] The estimated prevalence in the general population is 54 per 100,000. [32]

1.2.2 Etiology

Pathophysiology is unknown but suggested aetiological factors include repetitive mechanical injury (including eye rubbing), oxidative damage, genetic and immunological factors. [2] In addition, hormonal factors have been shown to play a potential role. [3-5] A number of investigators have reported that hypothyroidism may play a function in the exacerbation or even initiation of keratoconus. [3,4] Others have demonstrated a possible role of tear fluid thyroxine in keratoconus development. [5] Certainly, thyroid hormones are known to be important in corneal dehydration and transparency during embryonic development. [33] Conrad et al. have reported expression of thyroxine receptor A (THRA) mRNA in all layers of embryonic chicken corneas, with keratocytes and endothelial cells expressing thyroxine receptor B (THRB) mRNA. [34]

Corneal tissue degradation in keratoconus involves the expression of inflammatory mediators, such as pro-inflammatory cytokines, cell adhesion molecules, and matrix metalloproteinases.

Although keratoconus has been primarily defined as a non-inflammatory corneal disease, it has been reported that inflammatory molecules such as interleukin 1α and tumor necrosis factor α are over-expressed in keratoconus corneas as well as in the tear film of patients with keratoconus. [6-8]

1.2.3 Clinical features and diagnosis

Clinical features of keratoconus includes progressive corneal thinning and corneal protrusion, thereby inducing high myopia and irregular astigmatism, which severely affect visual acuity. In order to achieve sufficient visual restoration, approximately

20% of the keratoconus patients require penetrating keratoplasty after two decades. [35] Computer-assisted corneal topography is recognized as the “gold standard” for the diagnosis of keratoconus. [36] Placido-based modalities or Scheimpflug camera-based systems are important for the diagnosis of keratoconus, which facilitate an accurate analysis of both anterior and posterior corneal surface, thereby providing valuable topographic and pachymetric data. [37,38]

1.2.4 Treatment

In early stages of keratoconus, soft contact lenses are employed. As the condition progresses, rigid, gas-permeable (RGP) lenses are commonly accepted. But none of the contact lenses arrest progression of the condition. [39]

Corneal collagen crosslinking with riboflavin, also known as CXL and CCL, involves a one-time application of riboflavin solution to the eye that is activated by illumination with UVA light for approximately 30 minutes. [40,41] The riboflavin causes new bonds to form across adjacent collagen strands in the stromal layer of the cornea, which recovers and preserves some of the cornea's mechanical strength.

Keratoconus is the most common disease for conducting a penetrating keratoplasty, generally accounting for about a quarter of such procedures. [42] A recent surgical alternative to corneal transplant is the insertion of intrastromal corneal ring segments. The segments push out against the curvature of the cornea, flattening the peak of the cone and returning it to a more natural shape. The procedure involves no removal of eye tissue. [43]

1.3 Hormones

1.3.1 Thyroxine

Triiodothyronine (T_3) and thyroxine (T_4), are tyrosine-based hormones produced by the thyroid gland that are primarily responsible for regulation of metabolism. The major form of thyroid hormone in the blood is thyroxine. Thyroxine is essential to proper development and differentiation of all cells of the human body, which regulates protein, fat, and carbohydrate metabolism, affecting how human cells use energetic compounds. A number of physiological and pathological stimuli influence thyroid hormone synthesis.

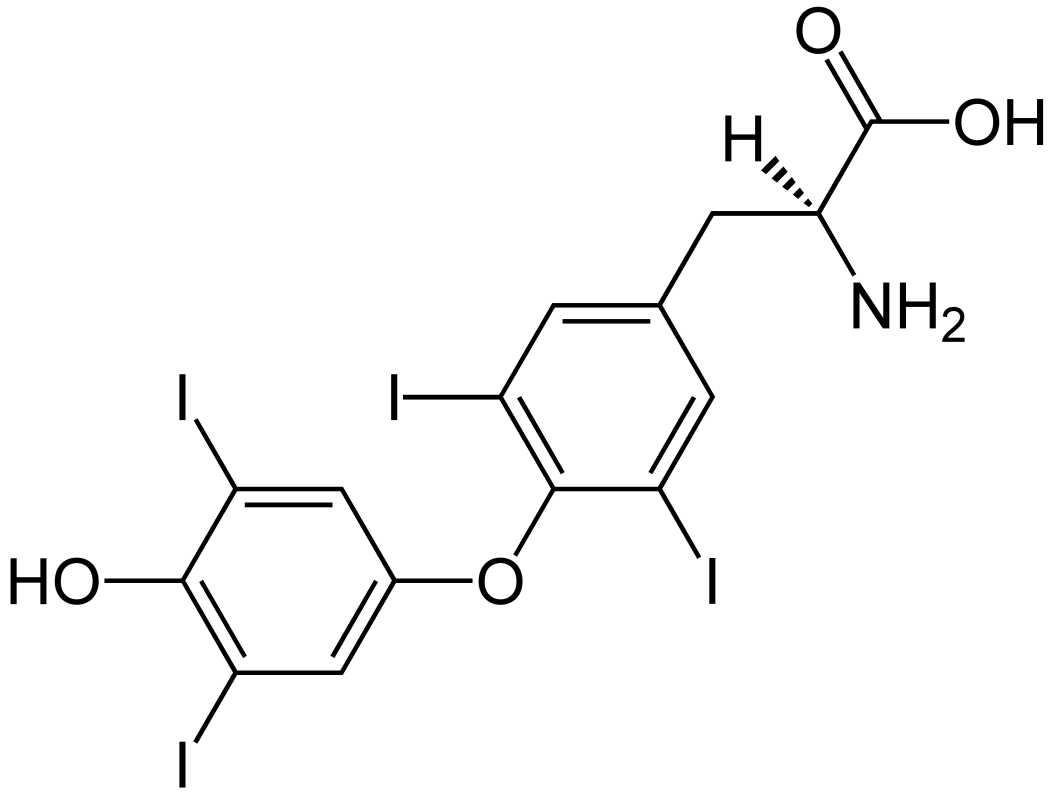

Figure 2. Thyroxine (3,5,3',5'-tetraiodothyronine)

1.3.2 Effect of thyroxine on the cornea

Although the etiology of keratoconus has not been clearly clarified, a number of investigators have reported that hypothyroidism may play a function in the exacerbation or even initiation of keratoconus. [3,4] Others have demonstrated a possible role of tear fluid thyroxine in keratoconus development. [5] Certainly, thyroid hormones

are known to be important in corneal dehydration and transparency during embryonic development. [33] Thyroxine receptor A (THRA) mRNA was found in all layers of embryonic chicken corneas, with keratocytes and endothelial cells expressing thyroxine receptor B (THRB) mRNA.[34]

1.3.3 Hydrocortisone

Cortisol is a steroid hormone, more specifically a glucocorticoid, produced by the zona fasciculata of the adrenal cortex. [44] Hydrocortisone is an identical molecule to cortisol, which can be used to treat people who lack adequate stores of endogenous cortisol. It is released in response to stress and a low level of blood glucocorticoid. Its primary functions are to increase blood sugar through gluconeogenesis, suppress the immune system, and aid with fat, protein, and carbohydrate metabolisms. [44] It also decreases bone formation. [45]

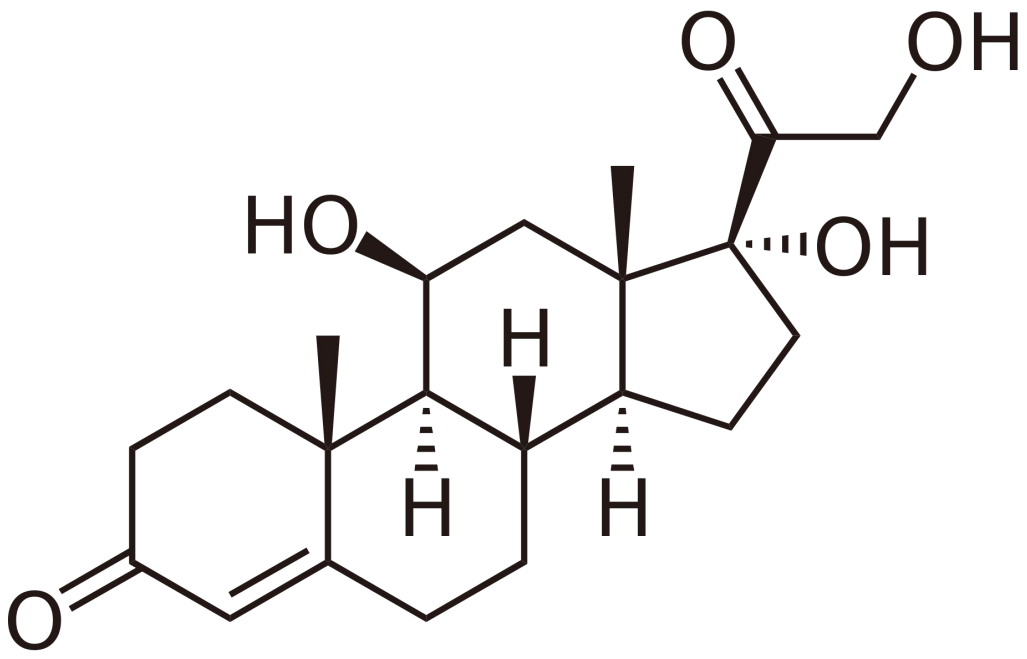

FIgure 3. Hydrocortisone ((11β)-11,17,21-trihydroxypregn-4-ene-3,20-dione)

1.3.4 Effect of hydrocortisone on the cornea

Hydrocortisone down-regulates the expression of Toll-like receptor-2 and -4 by corneal keratocytes *in vitro*, [46] thereby modulating the innate corneal immune system, since Toll-like receptors stimulate the pro-inflammatory cytokines and chemokines,

such as TNF-α, IL-6, IL-8, IL-18, and monocyte chemotactic protein-1. [47,48] The release of IL-6 and IL-8 is inhibited by hydrocortisone *in vitro*. [49]

1.4 Photodynamic Therapy

1.4.1 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 [50] 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 [51] 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 [52] and infectious disease [53] treatment. It has efficient cytotoxicity, but limited damage to the surrounding normal cells or tissues.

1.4.2 Photochemistry of PDT

The photochemical reaction is represented at the Jablonski diagram. [54] 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). [52, 55]

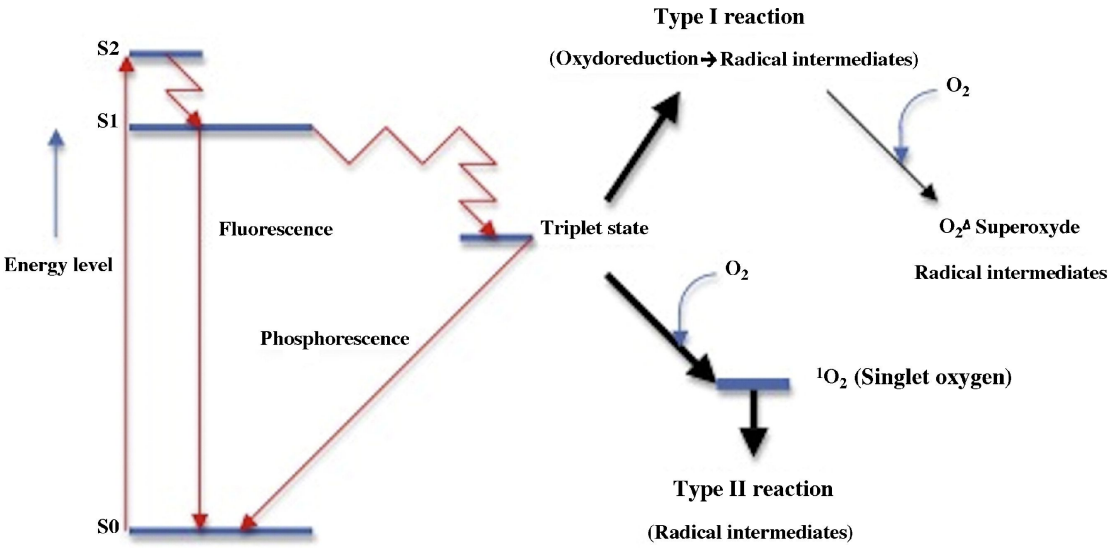

Figure 4. Jablonski diagram representing the excitation and relaxation of a photosensitizer (PS), with type I and type II photoreactions.

S0 = ground singlet state; S1 = first excited singlet state; S2 = second excited singlet state. [54]

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 [56]. 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, [57-59] but also seems to be the major pathway in reduction of organism damage in antimicrobial PDT. [61-63]

1.4.3 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). [64]

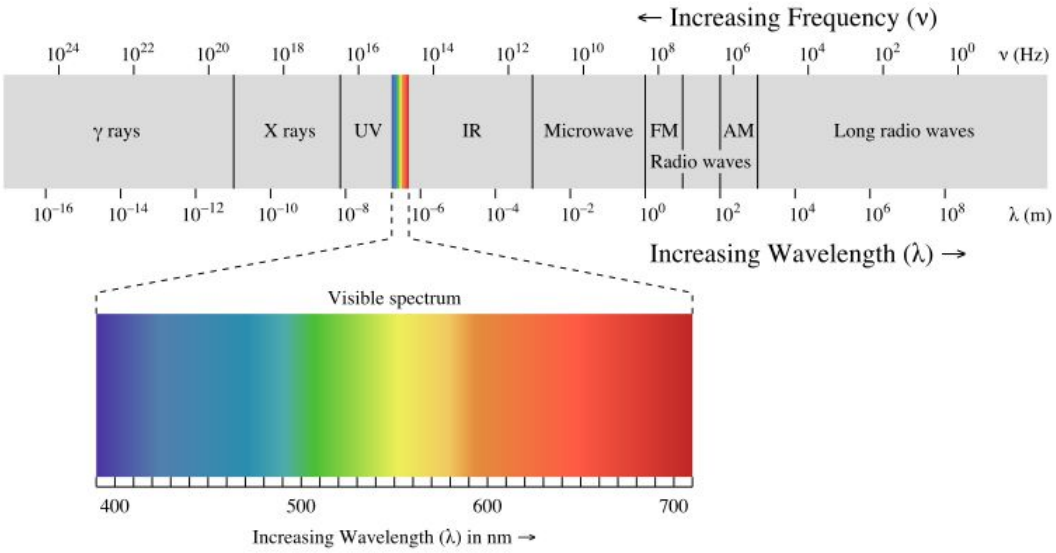

Figure 3. http://en.wikipedia.org/wiki/File:EM_spectrum.svg

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. [65]

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. [66,67] 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. [68] 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. [69,70]

1.4.4 Photosensitizers

The majority of Photosensitizers(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. [71] Therefore, the PS is considered to be a critical component in PDT procedures.

Since the first observation of tissue photochemical sensitization reported by Raab [50] 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. 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. [72,73] The fact that different generations of PSs exist does not mean that newer generation drugs in clinical application are better than older drugs. [71]

Riboflavin, also known as vitamin B2 and is the vitamin formerly known as G, [74] is an easily absorbed colored micronutrient with a key role in maintaining health in humans and other animals. Riboflavin in combination with UV light has been shown to be effective in reducing the ability of harmful pathogens found in blood products to cause disease. [75-78] Recently, riboflavin has been used in a new treatment to slow or stop the progression of the corneal disorder keratoconus, which is called corneal collagen crosslinking (CXL). In corneal crosslinking, riboflavin drops are applied to the patient's corneal surface. Once the riboflavin has penetrated through the cornea, ultraviolet A light therapy is applied. This induces collagen crosslinking, which increases the tensile strength of the cornea. [40,41]

1.5 Thesis aims

Keratoconus is a multi-factorial disease causing corneal degeneration, thinning and secondary ectasia, which can severely degrade visual performance. Its pathophysiology is unknown, but suggested aetiological factors include oxidative damage, repetitive mechanical injury, immunological factors and genetic factors. In addition, hormonal factors have been shown to play a potential role. 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 keratoconus keratocytes.

The *purpose* of this study was:

- To investigate the impact of thyroxine and hydrocortisone on the viability, proliferation and apoptosis of human keratocytes *in vitro* in normal and keratoconus corneas.
- To investigate the impact of thyroxine and hydrocortisone on the secretion of growth factors and interleukins by normal human keratocytes and keratoconus keratocytes *in vitro*.
- To determine the impact of riboflavin-UVA-PDT on viability, apoptosis, proliferation and activation of human keratoconus keratocytes, *in vitro.*
- To determine the impact of crosslinking on growth factor and interleukin secretion of human keratoconus keratocytes, 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). Thyroxine and hydrocortisone were obtained from Sigma-Aldrich (Hamburg, Germany). 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™ 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), IL-1β， EGF, MMP-9, MIP1α, IL-6, IL-8, TNFα were from BD Biosciences (Heidelberg, Germany), and TGFβ1 was from Gen-Probe Incorporated (San Diego, USA).

2.2 Isolation of primary human keratocytes

Human corneas were obtained from the Saarland University Hospital LIONS Eye Bank and Department of Ophthalmology, Saarland University. Human keratocytes were isolated as described previously. [79,80]

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.

2.3 Hormone treatment

Hydrocortisone was dissolved in absolute ethanol. Final dilution was carried out in DMEM/F-12 HAM obtaining a solution containing 20µg/ml. Thyroxine was diluted with DMEM/F-12 HAM obtaining a solution containing 50µg/ml. These were divided into aliquots containing the volume needed daily, kept frozen, and thawed only once on the day of use. The doses of both hormones used in this experiment were 20µg/ml, 10µg/ml, and 1µg/ml, respectively. In order to avoid the effect of ethanol in cell culture among different groups, contents of ethanol in each concentration of hydrocortisone were kept the same.

Before starting hormone treatment, culture medium was replaced with DMEM/F-12 HAM with 10% FBS. Different concentrations of hormones were added, as mentioned above, as well as cells seeded for 24 hours, and cultures were returned to the incubator for 24 hours.

2.4 Photodynamic Therapy (PDT) of primary human keratocytes

Human keratocytes were seeded in tissue culture plates and were allowed to grow for 48 hours before photodynamic treatment. For viability, apoptosis, proliferation, CD34 and alpha-SMA expression and cytokine measurements, 0.5% and 0.1% concentrations of riboflavin-5-phosphate were diluted in 20% Dextran-PBS. Cells were washed with PBS once before that the above riboflavin solution was added. Then, the cells were exposed directly to UVA light (370 nm) for 4 minutes 10 seconds

with an irradiation dose of 8 mW/cm^2 (2 J/cm^2). Following illumination, cells were washed twice with PBS, fed with culture medium and cultivated at 37 °C for twenty-four hours before measurements.

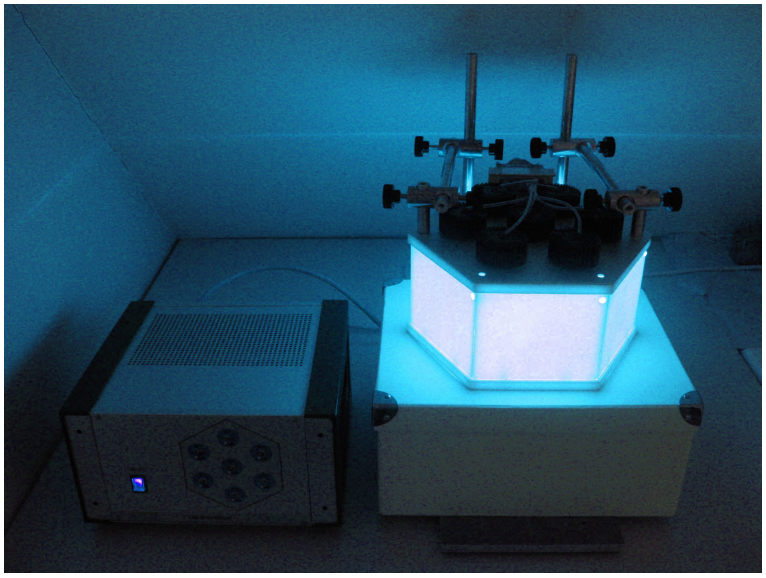

Figure 5. Illumination box (λ = 370 nm, 2 J/cm^2)

2.5 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).

2.6 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).

2.7 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).

2.8 Expression of CD34 and α-SMA of keratocytes

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).

2.9 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 IL-1β, IL-6, IL-8, FGFb, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα 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: EGF: 250-7.8 pg/mL, FGFb: 1000-8pg/mL, HGF: 8000-60 pg/mL, IL-6: 600-10 pg/mL， TGF β1: 2000-16 pg/mL, IL-1β: 1000-8 pg/mL, MMP9: 2000-16 pg/mL, TNFα: 1000-8 pg/mL, MIP1α: 500-4 pg/mL. Measured concentrations below the above values were considered as zero. The growth factor concentrations were quantified by using a human recombinant IL-1β, IL-6, IL-8, FGFb, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα 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, [80] 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.

2.10 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 Effects of thyroxine and hydrocortisone on human keratocytes in normal and keratoconus corneas *in vitro.*

Cell proliferation of NHPK increased significantly 24h after treatment with thyroxine from concentration of 1μg/ml and higher ($p<0.01$). Specifically, cell proliferation of NHPK increased by 51%, 46%, 53% at a concentration of 1μg/ml 10μg/ml and 20μg/ml, respectively. Significant increasing of proliferation in KHPK was only detected in 20μg/ml thyroxine group (increased by 67%) ($p<0.05$) **(Figure 5)**.

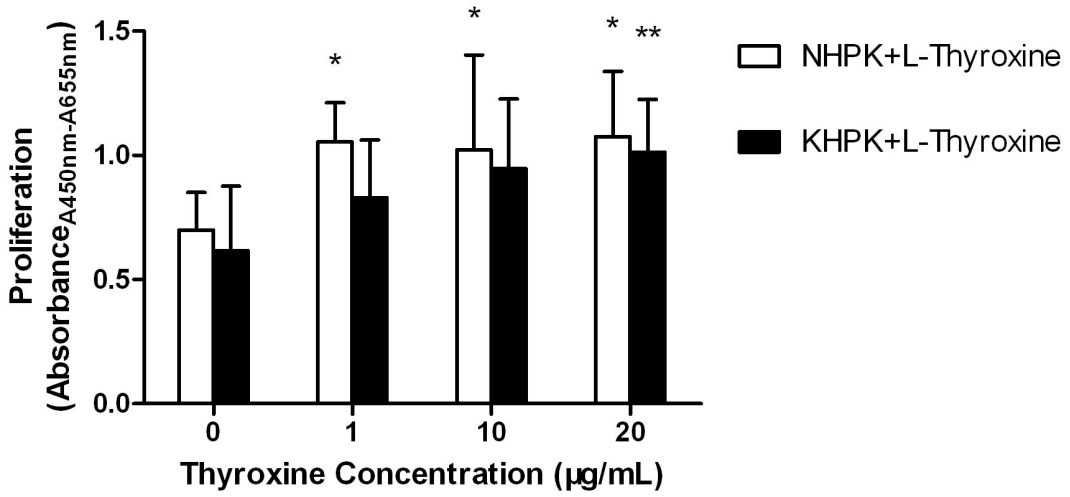

Figure 5. **Proliferation of NHPK and KHPK 24h after treatment with L-thyroxine *in vitro*.**

Cell proliferation of NHPK increased significantly 24h after treatment with 1ng/ml FGFb ($p<0.01$) and 2ng/ml TGFβ1 ($p<0.01$), while proliferation of KHPK decreased significantly 24h after treatment with the same concentration performed on NHPK ($p<0.01$, $p<0.01$) **(Figure 6).**

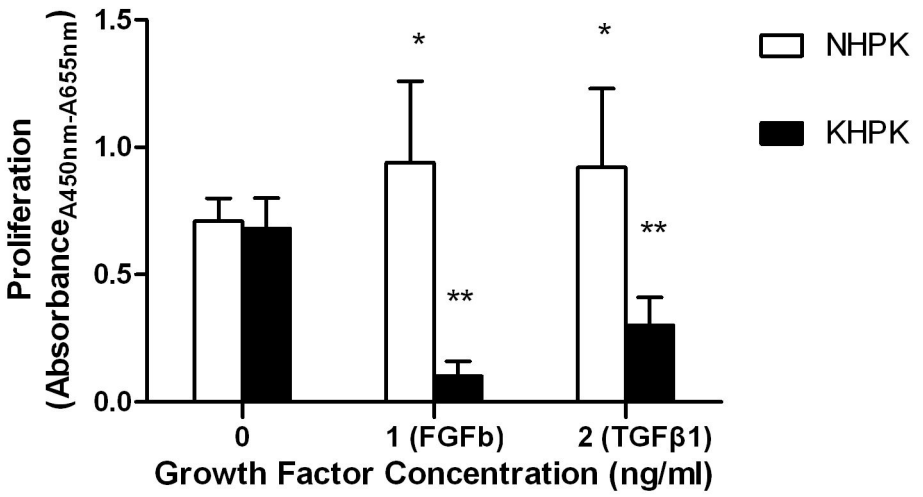

Figure 6. Proliferation of NHPK and KHPK 24h after treatment with FGFb and TGFβ1 in vitro.

We could not detect any changes in the proliferation of both NHPK and KHPK 24h after treatment with hydrocortisone ($p>0.05$). Twenty-four hours after hormone treatment, we did not observe any changes in cell viability of NHPK and KHPK for both thyroxine and hydrocortisone ($p>0.05$). Similarly, there were no changes observed in cell apoptosis of NHPK and KHPK after treatment with thyroxine and hydrocortisone (*$p>0.05$*) (data not shown).

3.2 Growth factor and interleukin secretion by human normal keratocytes and keratoconus keratocytes after hormone treatment *in vitro*

Cytokine secretion of normal keratocytes 5 h after hormone treatment

The mean concentration of **IL-6** 5 h after hormone treatment, using 10µg/mL L-Thyroxine or 10µg/mL hydrocortisone was significantly lower than that in the untreated group ($p=0.042$ and $p<0.001$, respectively).

The mean **IL-8** concentration after incubation with 10µg/mL hydrocortisone was significantly higher than that in the untreated group ($p<0.001$). However, we could not detect any changes following treatment with 10µg/mL L-Thyroxine ($p=0.336$).

The mean **FGFb** concentration decreased after treatment with 10µg/mL hydrocortisone (p=0.0019), whereas there were no changes detected following treatment with 10µg/mL L-Thyroxine (p=0.22).

We could not detect any changes in the secretion of **HGF** or **TGFβ1** at any of the examined groups 5 h following hormone treatment, compared to untreated controls (p>0.119).

The secretion of **IL-1β, EGF, MIP1α, MMP-9** and **TNFα** was below the detection limit in the treated and untreated cell cultures 5 h after treatment.

The concentrations of IL-1β, IL-6, IL-8, FGFb, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα secreted by normal keratocytes 5 h after hormone treatment are summarized in **Figure 7** and **Table 1**.

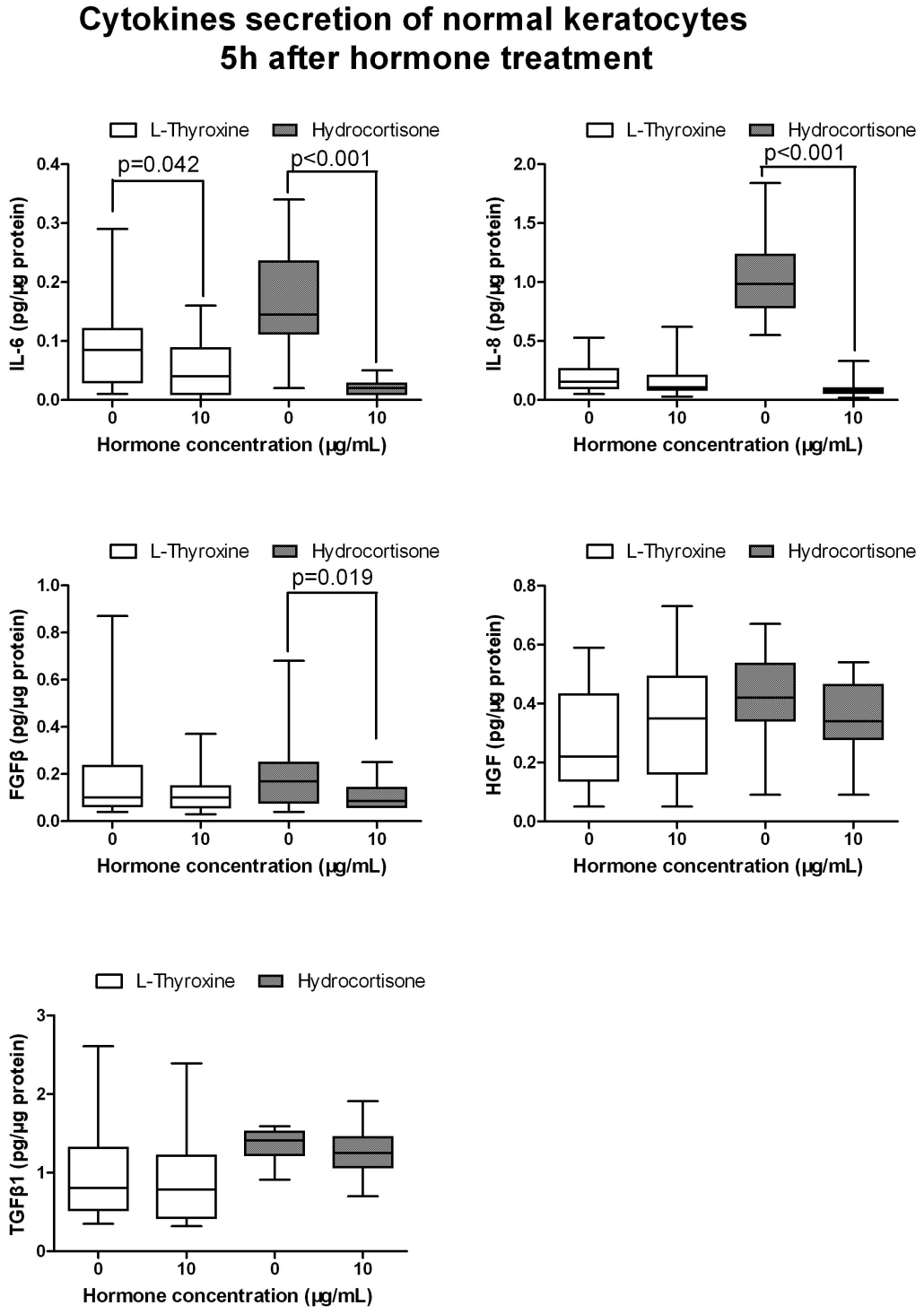

Figure 7. Cytokine secretion of normal keratocytes 5 h after hormone treatment

	Keratocytes + none L-Thyroxine	Keratocytes+ 10µg/mL L-Thyroxine	Keratocytes + none hydrocortisone	Keratocytes + 10 µg/mL hydrocortisone	* p-value	** p-value
IL-6	0.098±0.083	0.053±0.048	0.163±0.081	0.019±0.013	***0.042***	***<0.001***
IL-8	0.206±0.147	0.161±0.146	1.02±0.314	0.088±0.065	0.336	***<0.001***
FGFb	0.194±0.222	0.128±0.093	0.214±0.179	0.111±0.059	0.227	***0.002***
HGF	0.278±0.182	0.343±0.208	0.421±0.142	0.355±0.121	0.299	0.119
TGFβ1	0.977±0.597	0.892±0.568	1.358±0.189	1.246±0.309	0.649	0.175
IL-1β	n.d.	n.d.	n.d.	n.d.	n/a	n/a
EGF	n.d.	n.d.	n.d.	n.d.	n/a	n/a
MIP1α	n.d.	n.d.	n.d.	n.d.	n/a	n/a
MMP-9	n.d.	n.d.	n.d.	n.d.	n/a	n/a
TNFα	n.d.	n.d.	n.d.	n.d.	n/a	n/a

Table 1. Concentration (pg/µg protein) of different cytokines in the supernatant of normal keratocyte cultures 5 h after hormone treatment.

Values indicate mean± SD.

* p-values indicate the difference between "Keratocytes + none L-Thyroxine" versus "Keratocytes+ 10µg/mL L-Thyroxine" groups.

** p-values indicate the difference between "Keratocytes + none hydrocortisone" versus "Keratocytes + 10 µg/mL hydrocortisone" groups.

Significant values are shown in bold (one-way ANOVA test).

n.d. = not detectable

n/a = not applicable

Cytokine secretion of keratoconus keratocytes 5 h after hormone treatment

The mean **IL-6** concentration significantly increased following treatment with 10μg/mL hydrocortisone (p<0.001). However, we could not detect any changes following treatment with 10μg/mL L-Thyroxine (p=0.949).

The mean **IL-8** and **TNFα** concentration was significantly higher 5 h after treatment with 10μg/mL (p=0.003 and p=0.02, respectively) and significantly lower following treatment with 10μg/mL hydrocortisone (p=0.002 and p=0.016, respectively).

We could not detect changes in the secretion of **MIP1α** or **TGFβ1** at any of the examined groups 5 h following hormone treatment, compared to untreated controls (p>0.183).

The secretion of **IL-1β, EGF, FGFb, MMP-9** and **HGF** was below the detection limit in the treated and untreated cell cultures 5 h after treatment.

The concentration of IL-1β, IL-6, IL-8, FGFb, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα secreted by keratoconus keratocytes 5 h after hormone treatment are summarized in **Figure 8** and **Table 2** .

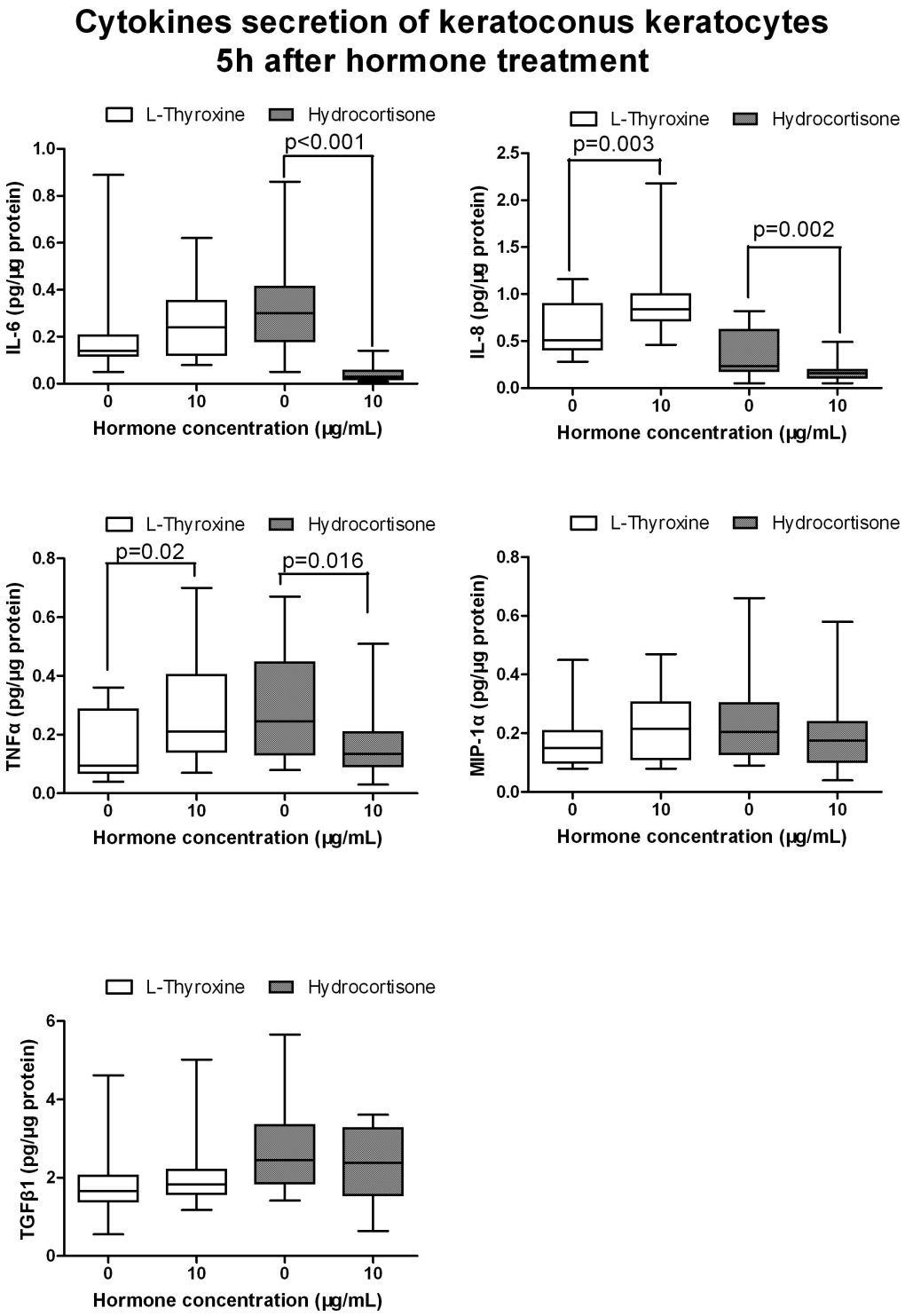

Figure 8. Cytokine secretion of keratoconus keratocytes 5 h after hormone treatment

	Keratocytes + none L-Thyroxine	Keratocytes+ 10µg/mL L-Thyroxine	Keratocytes + none hydrocortisone	Keratocytes + 10 µg/mL hydrocortisone	* p-value	** p-value
IL-6	0.249±0.236	0.253±0.152	0.31±0.193	0.042±0.038	0.949	***<0.001***
IL-8	0.592±0.279	0.943±0.418	0.359±0.24	0.168±0.09	***0.003***	***0.002***
TNFα	0.158±0.11	0.276±0.188	0.298±0.182	0.174±0.126	***0.02***	***0.016***
MIP1α	0.179±0.102	0.227±0.127	0.251±0.158	0.189±0.125	0.2	0.183
TGFβ1	1.94±0.94	2.07±0.91	2.68±1.045	2.392±0.965	0.669	0.371
FGFb	n.d.	n.d.	n.d.	n.d.	n/a	n/a
HGF	n.d.	n.d.	n.d.	n.d.	n/a	n/a
EGF	n.d.	n.d.	n.d.	n.d.	n/a	n/a
IL-1β	n.d.	n.d.	n.d.	n.d.	n/a	n/a
MMP-9	n.d.	n.d.	n.d.	n.d.	n/a	n/a

Table 2. Concentration (pg/µg protein) of different cytokines in the supernatant of keratoconus keratocyte cultures 5 h after hormone treatment.

Values indicate mean± SD.

* p-values indicate the difference between "Keratocytes + none L-Thyroxine" versus "Keratocytes+ 10µg/mL L-Thyroxine" groups.

** p-values indicate the difference between "Keratocytes + none hydrocortisone" versus "Keratocytes + 10 µg/mL hydrocortisone" groups.

Significant values are shown in bold (one-way ANOVA test).

n.d. = not detectable

n/a = not applicable

Cytokine secretion of normal keratocytes 24 h after hormone treatment

The mean **IL-6, IL-8**, **EGF** and **TGFβ1** concentration was significantly decreased after treatment with 10μg/mL hydrocortisone ($p<0.001$, $p=0.017$, $p=0.02$ and $p=0.019$, respectively), whereas no changes were observed following treatment with 10μg/mL L-Thyroxine ($p=0.736$, $p=0.301$, $p=0.297$ and $p=0.651$, respectively).

The mean **HGF** concentration after treatment with 10μg/mL L-Thyroxine, was significantly increased ($p=0.038$), while there were no changes following treatment with 10μg/mL hydrocortisone ($p=0.783$).

We could not detect changes in the secretion of **TNFα** at any of the examined groups 24 h following hormone treatment, compared to untreated controls ($p>0.534$).

The secretion of **IL-1β, MIP1α, MMP-9** and **FGFb** was below the detection limit in the treated and untreated cell cultures 24 h after treatment.

The concentrations of IL-1β, IL-6, IL-8, FGFb, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα secreted by normal keratocytes 24 h after hormone treatment are summarized in **Figure 9** and **Table 3**.

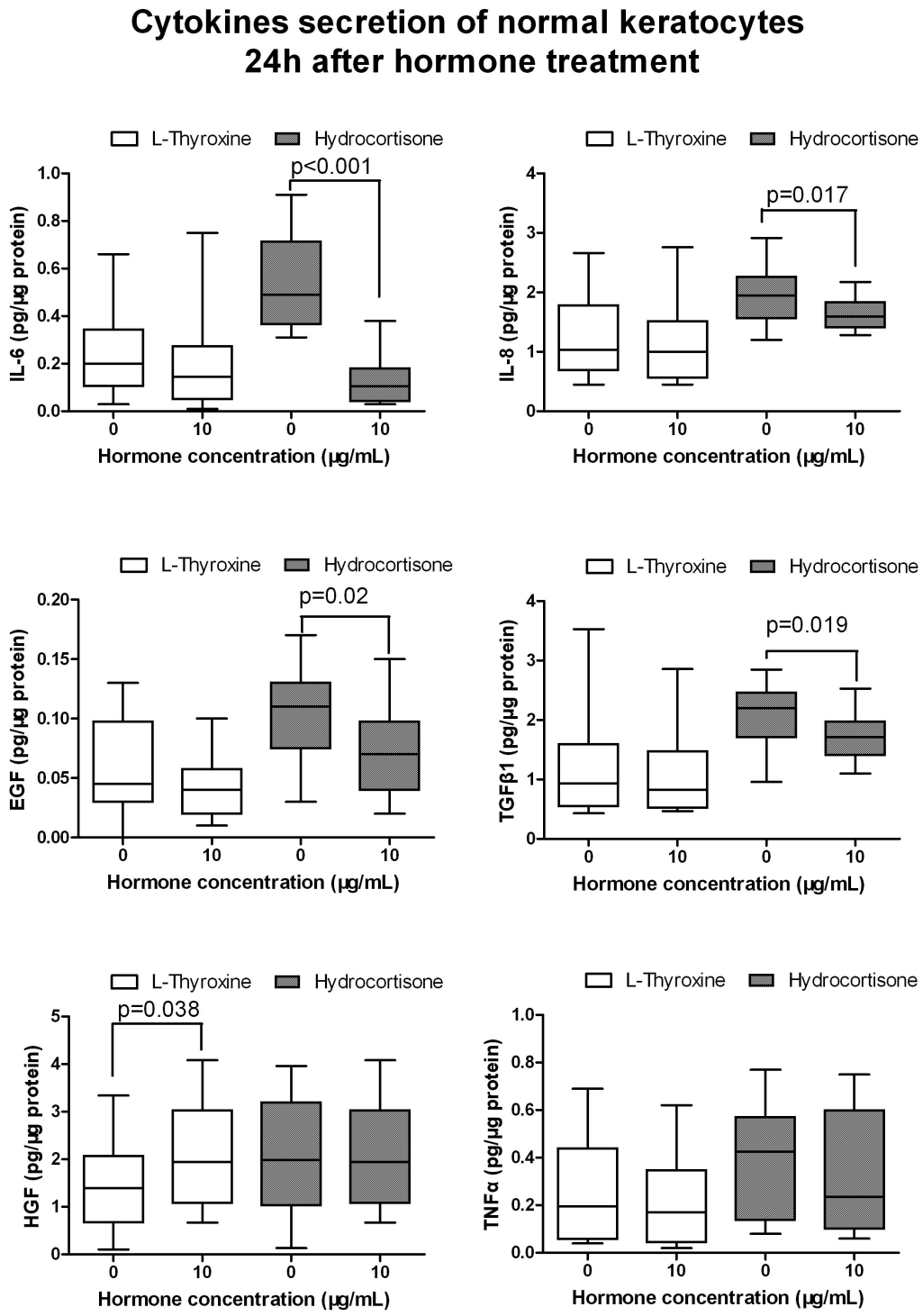

Figure 9. Cytokine secretion of normal keratocytes 24 h after hormone treatment

	Keratocytes + none L-Thyroxine	Keratocytes+ 10µg/mL L-Thyroxine	Keratocytes + none hydrocortisone	Keratocytes + 10 µg/mL hydrocortisone	* p-value	** p-value
IL-6	0.24±0.177	0.182±0.183	0.543±0.198	0.122±0.089	0.301	***<0.001***
IL-8	1.228±0.648	1.158±0.664	1.941±0.446	1.648±0.272	0.736	***0.017***
EGF	0.057±0.041	0.045±0.027	0.102±0.039	0.073±0.036	0.297	***0.02***
TGFβ1	1.229±0.864	1.114±0.725	2.076±0.522	1.718±0.388	0.651	***0.019***
HGF	1.433±0.919	2.119±1.087	2.036±1.236	2.136±1.022	***0.038***	0.783
TNFα	0.248±0.196	0.223±0.191	0.384±0.234	0.335±0.255	0.679	0.534
FGFb	n.d.	n.d.	n.d.	n.d.	n/a	n/a
MIP1α	n.d.	n.d.	n.d.	n.d.	n/a	n/a
IL-1β	n.d.	n.d.	n.d.	n.d.	n/a	n/a
MMP-9	n.d.	n.d.	n.d.	n.d.	n/a	n/a

Table 3. Concentration (pg/µg protein) of different cytokines in the supernatant of normal keratocyte cultures 24 h after hormone treatment.

Values indicate mean± SD.

* p-values indicate the difference between "Keratocytes + none L-Thyroxine" versus "Keratocytes+ 10µg/mL L-Thyroxine" groups.

** p-values indicate the difference between "Keratocytes + none hydrocortisone" versus "Keratocytes + 10 µg/mL hydrocortisone" groups.

Significant values are shown in bold (one-way ANOVA test).

n.d. = not detectable

n/a = not applicable

Cytokine secretion of keratoconus keratocytes 24 h after hormone treatment

The mean **IL-6** and **TGFβ1** concentration was significantly decreased 24 h following treatment with 10μg/mL hydrocortisone ($p<0.001$ and $p<0.001$, respectively), whereas there were no changes observed after treatment with 10μg/mL L-Thyroxine ($p=0.433$ and $p=0.955$, respectively).

The mean **HGF** concentration was significantly increased after treatment with 10μg/mL L-Thyroxine ($p=0.005$), and decreased after treatment with 10μg/mL hydrocortisone ($p<0.001$).

We could not detect changes in the secretion of **IL-8** or **TNFα** at any of the examined groups 24 h following hormone treatment, compared to untreated controls ($p>0.417$).

The secretion of **IL-1β, MIP1α, MMP-9, EGF** and **FGFb** was below the detection limit in the treated and untreated cell cultures 24h after treatment.

The concentrations of IL-1β, IL-6, IL-8, FGFb, HGF, TGFβ1, EGF, MIP1α, MMP-9 and TNFα secreted by keratoconus keratocytes 24 h after hormone treatment are summarized in **Figure 10** and **Table 4**.

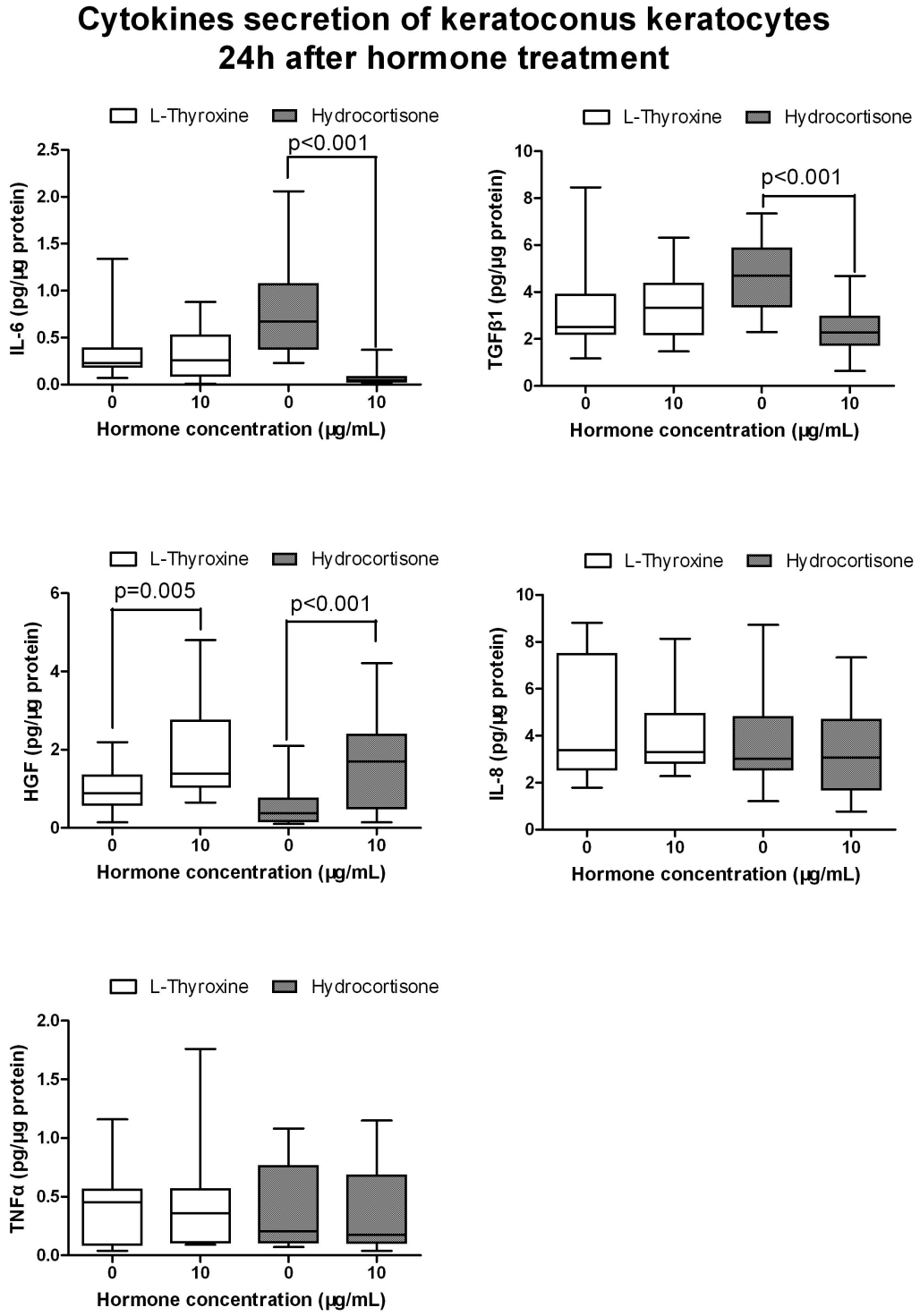

Figure 10. Cytokine secretion of keratoconus keratocytes 24 h after hormone treatment

	Keratocytes + none L-Thyroxine	Keratocytes+ 10µg/mL L-Thyroxine	Keratocytes + none hydrocortisone	Keratocytes + 10 µg/mL hydrocortisone	* p-value	** p-value
IL-6	0.387±0.368	0.308±0.252	0.777±0.476	0.081±0.088	0.433	***<0.001***
TGFβ1	3.317±1.894	3.347±1.431	4.693±1.46	2.355±1.075	0.955	***<0.001***
HGF	1.007±0.604	1.889±1.184	0.522±0.498	1.637±1.173	***0.005***	***<0.001***
IL-8	4.73±2.616	4.141±1.854	3.726±1.955	3.298±1.905	0.417	0.487
TNFα	0.44±0.353	0.409±0.397	0.424±0.37	0.379±0.373	0.799	0.783
EGF	n.d.	n.d.	n.d.	n.d.	n/a	n/a
FGFb	n.d.	n.d.	n.d.	n.d.	n/a	n/a
MIP1α	n.d.	n.d.	n.d.	n.d.	n/a	n/a
IL-1β	n.d.	n.d.	n.d.	n.d.	n/a	n/a
MMP-9	n.d.	n.d.	n.d.	n.d.	n/a	n/a

Table 4. Concentration (pg/µg protein) of different cytokines in the supernatant of keratoconus keratocyte cultures 24 h after hormone treatment.

Values indicate mean± SD.

* p-values indicate the difference between “Keratocytes + none L-Thyroxine” versus “Keratocytes+ 10µg/mL L-Thyroxine” groups.

** p-values indicate the difference between “Keratocytes + none hydrocortisone” versus “Keratocytes + 10 µg/mL hydrocortisone” groups.

Significant values are shown in bold (one-way ANOVA test).

n.d. = not detectable

n/a = not applicable

3.3 Viability, apoptosis, proliferation and activation of human keratoconus keratocytes after crosslinking/ riboflavin-UVA-photodynamic therapy (PDT)

KC keratocyte viability

Results of the alamarBlue® assay are shown in **Figure 11** (n=5). With the separate use of riboflavin or UVA-light illumination, viability of KC keratocytes did not change significantly compared to controls (p>0.34). Following riboflavin-UVA-PDT, using 0.1% riboflavin , KC keratocyte viability was significantly decreased (p=0.047), compared to controls.

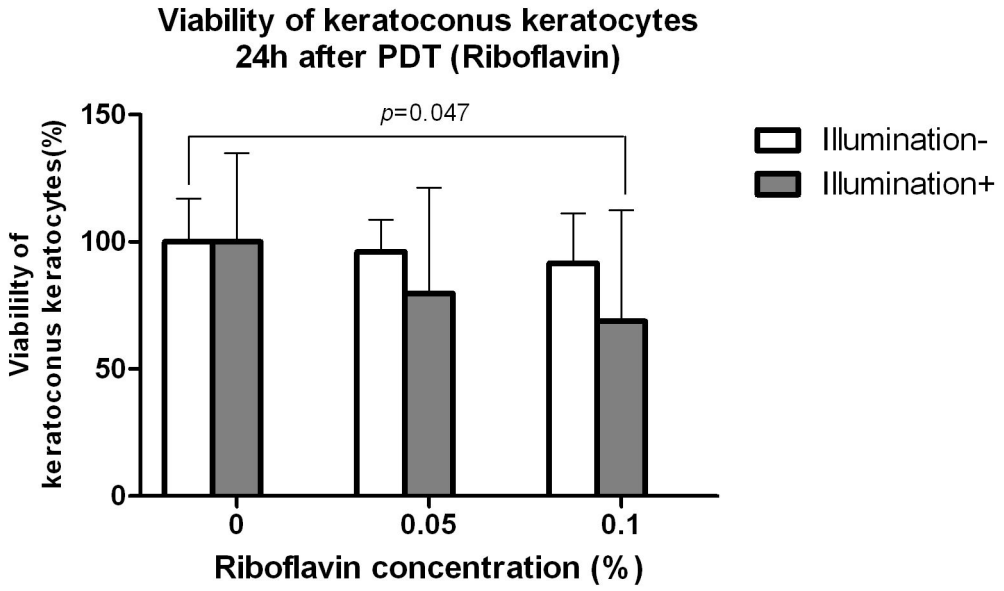

Figure 11. Viability of human KC keratocytes 24 hours following PDT.

KC keratocyte apoptosis

Figure 12 shows the percentage of apoptotic KC keratocytes 24 hours following crosslinking (n=5). Using the APO-DIRECT^{TM} kit, there was no significant difference in the percentage of apoptotic KC keratocytes using riboflavin or UVA-light illumination separately (p>0.26). However, using riboflavin-UVA-PDT, with 0.05% or 0.1% riboflavin, significantly increased the percentage of apoptotic KC keratocytes compared to controls (p=0.025 and p=0.01 respectively).

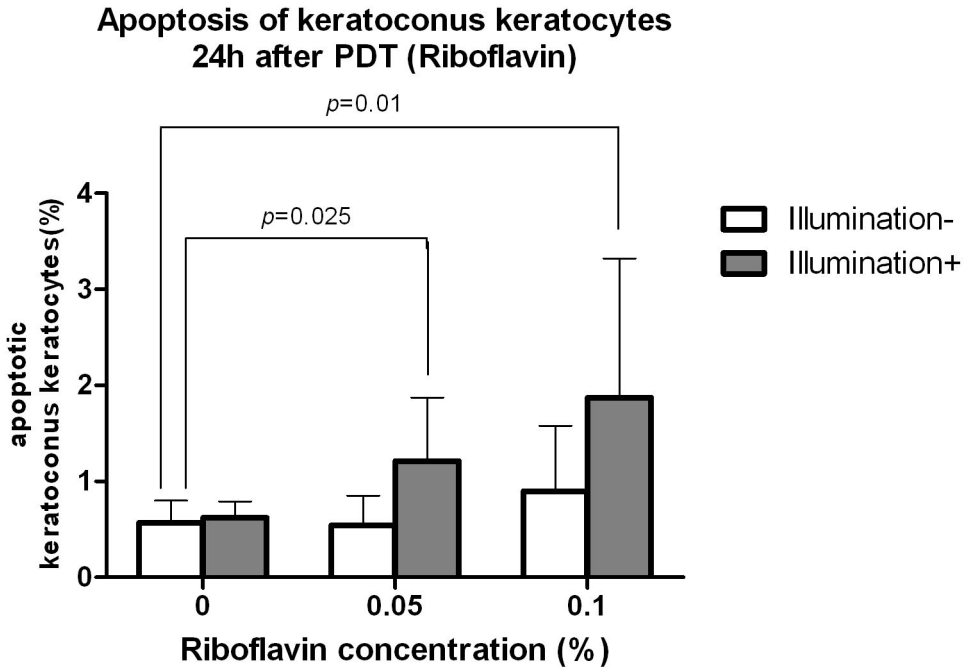

Figure 12. Percentage of apoptotic KC keratocytes 24 hours following PDT.

KC keratocyte proliferation

Proliferation of KC keratocytes is displayed in **Figure 13** (n=5). Using riboflavin or UVA light illumination separately, proliferation of KC keratocytes did not change significantly ($p>0.25$). Twenty-four hours after PDT, proliferation of KC keratocytes was inhibited significantly using 0.05% or 0.1% riboflavin concentrations ($p=0.009$ for both concentrations) compared to controls.

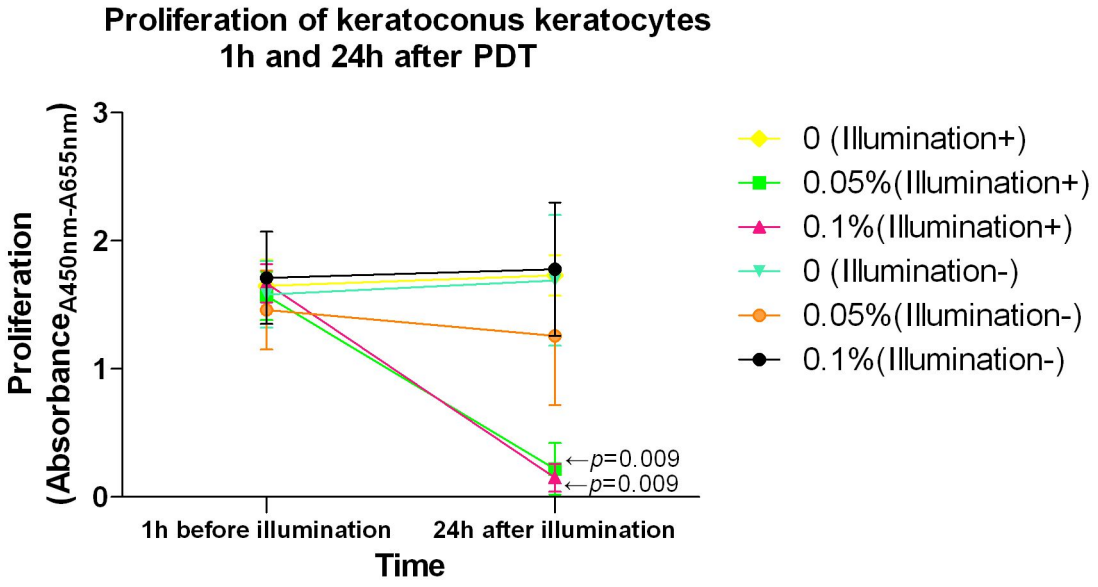

Figure 13. Proliferation of human KC keratocytes 24 hours after PDT.

CD34 expression of KC keratocytes

CD34 expression of KC keratocytes twenty-four hours following PDT is summarized in **Figure 14** (n=5). Using riboflavin, illumination only or using riboflavin-UVA-PDT resulted in no significant change in CD34 expression compared to controls (p>0.15)**.**

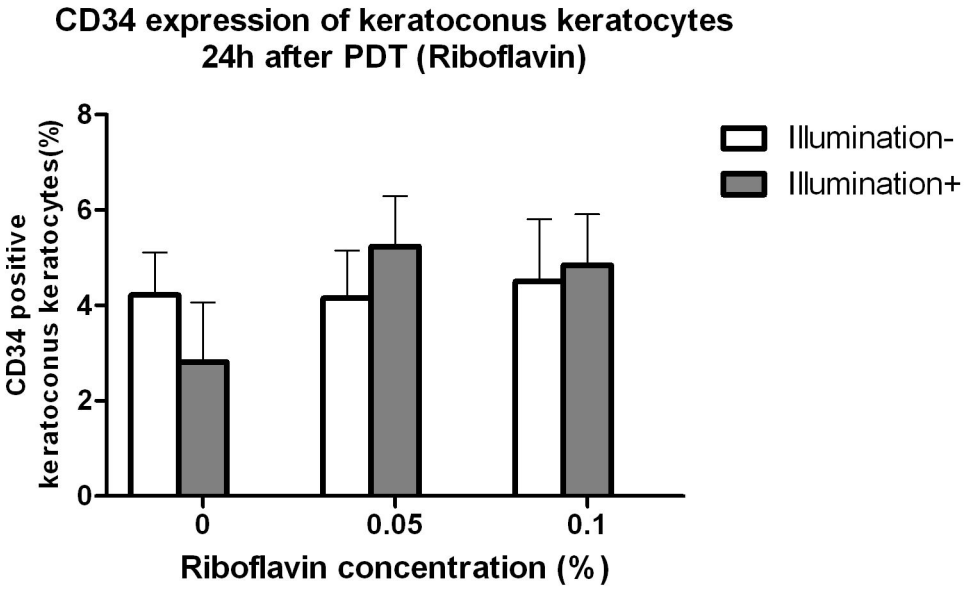

Figure 14. CD34 expression of keratoconus keratocytes 24 hours after PDT.

Alpha-SMA expression of KC keratocytes

Alpha-SMA expression in KC keratocytes twenty-four hours following PDT is summarized in **Figure 15** (n=5). There was no significant difference in the percentage of alpha-SMA positive KC keratocytes using riboflavin or UVA-light illumination separately (p>0.16). Using riboflavin-UVA-PDT, there was also no significant difference at 0.05% or 0.1% riboflavin (p=0.15 and p=0.06, respectively) in alpha-SMA expression in keratocytes compared to controls.

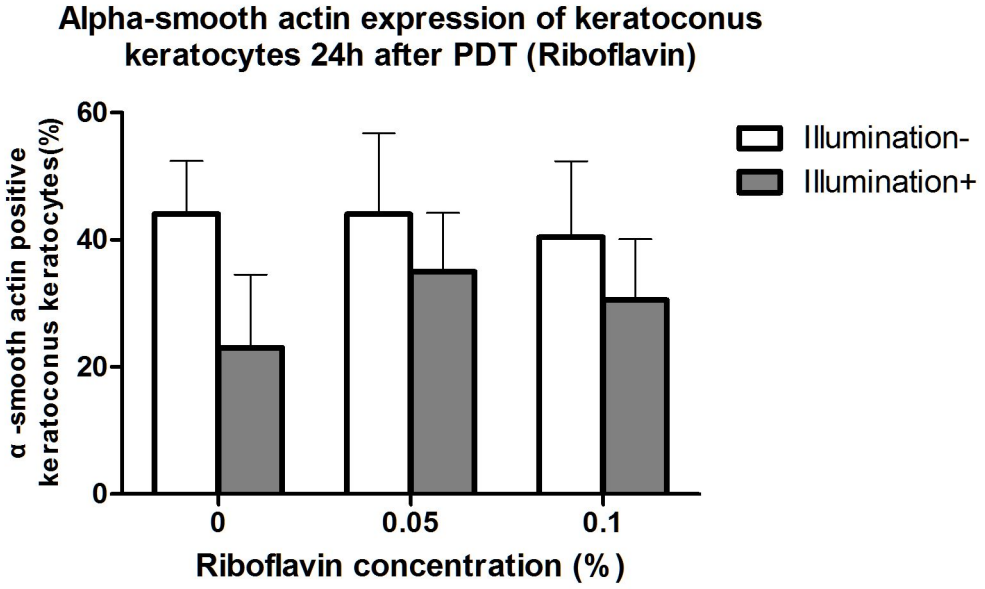

Figure 15. α-SMA expression of KC keratocytes 24 hours after PDT.

3.4 Growth factor and interleukin secretion of human keratoconus keratocytes after crosslinking / riboflavin-UVA-photodynamic treatment (PDT)

Growth factors secretions 5 hours after crosslinking

GFb, KGF, VEGF, HGF and TGFβ1 concentrations 5 hours after crosslinking are summarized in **Figure 16** and **Table 5**. The secretion of KGF was below the detection limit in the treated and untreated cell cultures 5 hours after treatment. With the separate use of riboflavin or UVA-light illumination, growth factor and interleukin secretion of keratoconus keratocytes did not differ significantly from untreated controls (p>0.34, p>0.08). Using 0.1% riboflavin and illumination, the mean FGFb

concentration was 6.32 ± 1.84 pg/ µg protein in the supernatant of the medium of KC keratocytes. This was significantly higher than FGFb concentration in untreated KC keratocyte cultures (3.28 ± 2.40 pg/µg protein; p=0.037). We could not detect changes in the secretion of VEGF, HGF, or TGFβ1 of KC keratocytes at any of the examined groups 5 hours following crosslinking, compared to controls (p>0.35).

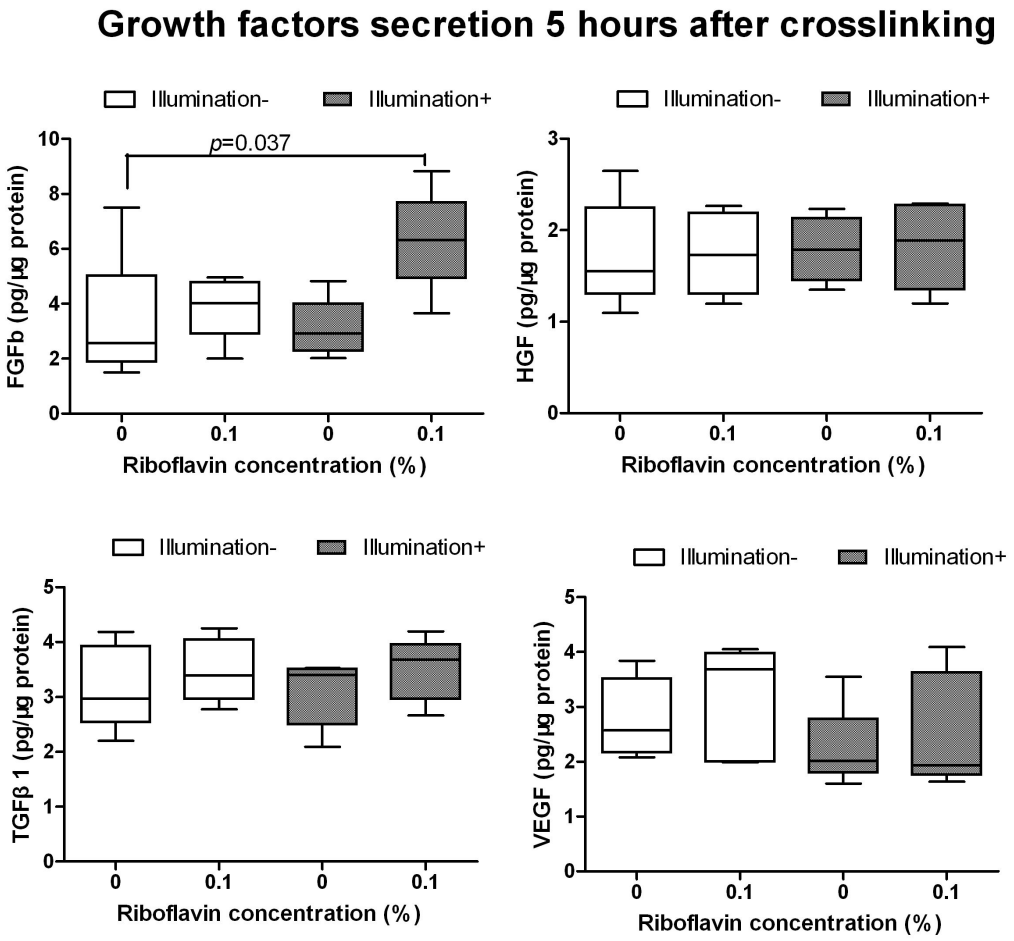

Figure 16. FGFb, HGF, TGFβ1 and VEGF secretion of keratocytes (mean pg/µg protein ± SD) *5 hours* following crosslinking.

	Keratocytes	Keratocytes + riboflavin	Keratocytes + 370nm	Keratocytes + riboflavin + 370nm	* p-value	** p-value	*** p-value
FGFb	3.28±2.40	3.89±1.15	3.10±1.06	6.32±1.84	0.34	0.6	***0.037***
HGF	1.73±0.58	1.74±0.45	1.79±0.36	1.83±0.48	0.92	0.6	0.75
TGFβ1	3.19±0.76	3.48±.58	3.09±0.61	3.51±0.57	0.47	0.75	0.35
VEGF	2.79±0.07	3.13±1.04	2.24±0.75	2.55±1.05	0.75	0.08	0.35
KGF	n.d.	n.d.	n.d.	n.d.	n/a	n/a	n/a

Table 5. Concentration (pg/μg protein) of different growth factors in the supernatant of keratoconus keratocyte cultures 5 hours after crosslinking. Values indicate mean± SD.

* p-values indicate the difference between "Keratocytes" versus "Keratocytes + Riboflavin" groups.

** p-values indicate the difference between "Keratocytes" versus "Keratocytes + 370 nm" groups.

*** p-values indicate the difference between "Keratocytes" versus "Keratocytes + Riboflavin + 370 nm" groups.

Significant values are shown in bold (Mann-Whitney U test).

n.d. = not detectable

n/a = not applicable

Interleukins secretions 5 hours after corsslinking

IL-1β, IL-6 and IL-8 concentrations 5 hours after crosslinking are summarized in **Figure 17** and **Table 6**. The secretion of IL-1β was below the detection limit in the treated and untreated cell cultures 5 hours after treatment. With the separate use of riboflavin or UVA-light illumination, growth factor and interleukin secretion of keratoconus keratocytes did not differ significantly from untreated controls ($p>0.47$, $p>0.47$). We could not detect changes in the secretion of IL-6, or IL-8 of KC keratocytes at any of the examined groups 5 hours following crosslinking, compared to controls ($p>0.35$).

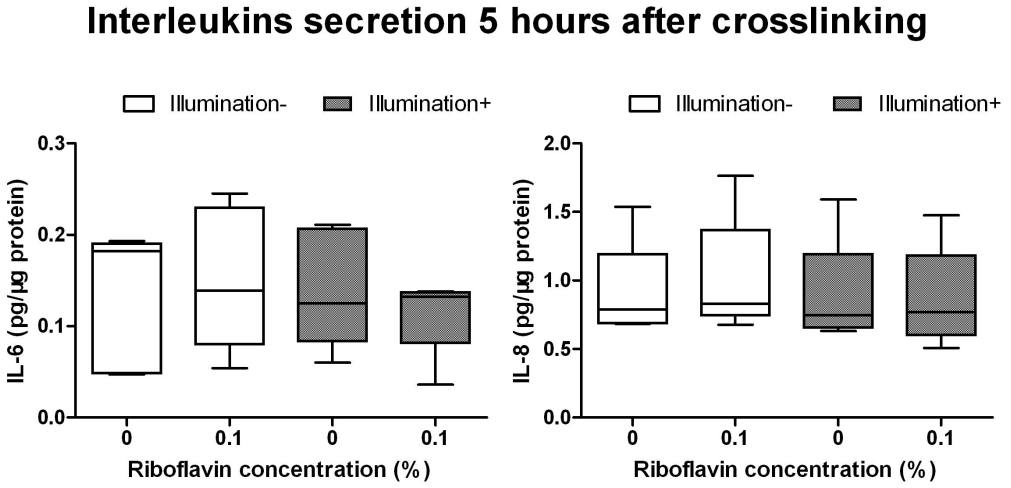

Figure 17. IL-6 and IL-8 secretion of keratocytes (mean pg/µg protein ± SD) *5 hours* following crosslinking.

	Keratocytes	Keratocytes + riboflavin	Keratocytes + 370nm	Keratocytes + riboflavin + 370nm	* p-value	** p-value	*** p-value
IL-1β	n.d.	n.d.	n.d.	n.d.	n/a	n/a	n/a
IL-6	0.13±0.08	0.15±0.08	0.14±0.06	0.11±0.04	0.47	0.47	0.35
IL-8	0.91±0.36	1.01±0.34	0.89±0.4	0.87±0.37	0.6	0.47	0.92

Table 6. Concentration (pg/µg protein) of different interleukins in the supernatant of keratoconus keratocyte cultures 5 hours after crosslinking. Values indicate mean± SD.
* p-values indicate the difference between "Keratocytes" versus "Keratocytes + Riboflavin" groups.
** p-values indicate the difference between "Keratocytes" versus "Keratocytes + 370 nm" groups.
*** p-values indicate the difference between "Keratocytes" versus "Keratocytes + Riboflavin + 370 nm" groups.
Significant values are shown in bold (Mann-Whitney U test).
n.d. = not detectable
n/a = not applicable

Growth factors secretions 24 hours after crosslinking

Figure 18 and **Table 7** display FGFb, KGF, VEGF, HGF and TGFβ1 concentrations 24 hours after crosslinking. KGF secretion was below the detection limit in the treated and untreated cell cultures 24 hours after crosslinking. With the separate use of riboflavin or UVA-light illumination, growth factor and interleukin secretion of keratoconus keratocytes did not change significantly compared to controls (p>0.12, p>0.35). 24 hours after crosslinking, FGFb, VEGF, HGF and TGFβ1 secretion also did not change significantly compared to untreated control KC keratocyte cultures (p>0.12).

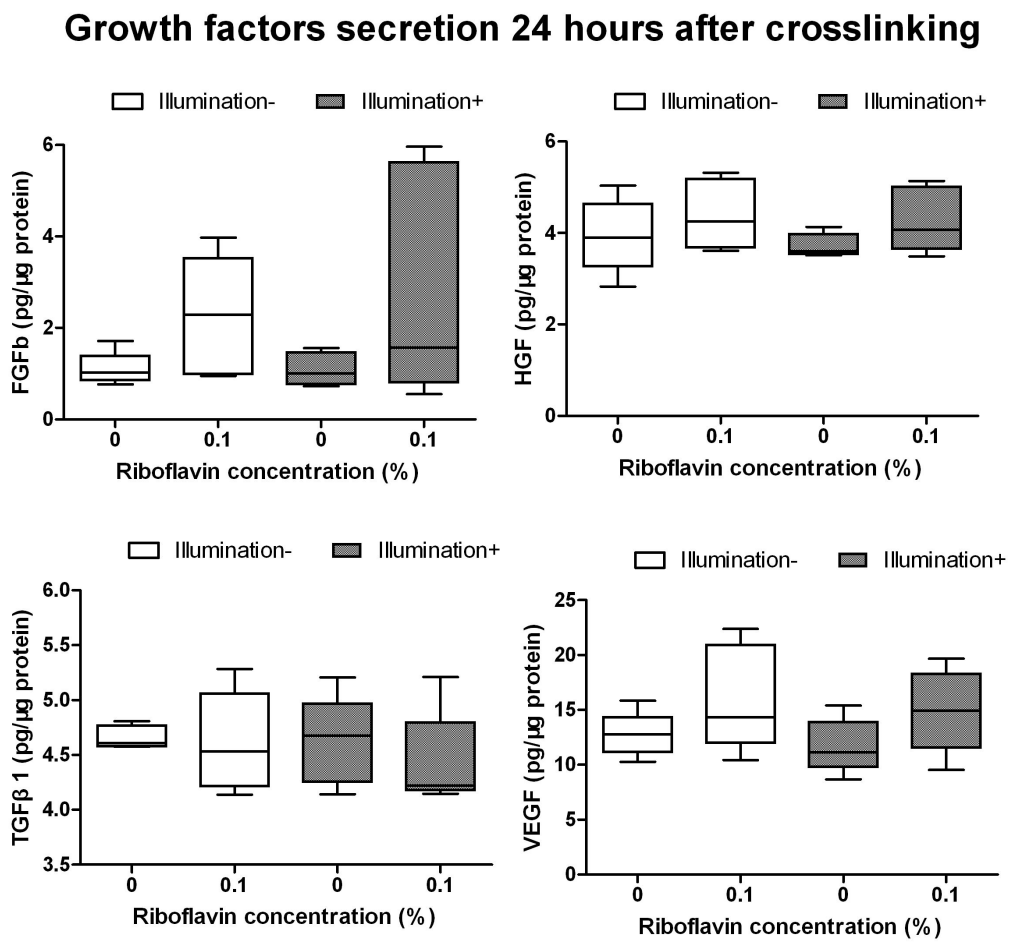

Figure 18. FGFb, HGF, TGFβ1 and VEGF secretion of keratocytes (mean pg/µg protein ± SD) *24 hours* following crosslinking.

	Keratocytes	Keratocytes + riboflavin	Keratocytes + 370nm	Keratocytes + riboflavin + 370nm	* p-value	** p-value	*** p-value
FGFb	1.1±0.36	2.26±1.3	1.09±0.36	2.88±2.53	0.12	0.75	0.3
HGF	3.94±0.8	4.39±0.77	3.72±0.26	4.28±0.71	0.35	0.35	0.6
TGFβ1	4.66±0.1	4.62±0.46	4.62±0.4	4.43±0.44	0.6	0.92	0.12
VEGF	12.75±2.01	16.02±4.82	11.7±2.47	14.92±3.8	0.18	0.35	0.25
KGF	n.d.	n.d.	n.d.	n.d.	n/a	n/a	n/a

Table 7. Concentration (pg/µg protein) of different cytokines in the supernatant of keratoconus keratocyte cultures 24 hours after crosslinking. Values indicate mean± SD.

* p-values indicate the difference between "Keratocytes" versus "Keratocytes + Riboflavin" groups.

** p-values indicate the difference between "Keratocytes" versus "Keratocytes + 370 nm" groups.

*** p-values indicate the difference between "Keratocytes" versus "Keratocytes + Riboflavin + 370 nm" groups.

Significant values are shown in bold (Mann-Whitney U test).

n.d. = not detectable

n/a = not applicable

Interleukins secretions 24 hours after crosslinking

Figure 19 and **Table 8** display IL-1β, IL-6 and IL-8 concentrations 24 hours after crosslinking. IL-1β secretion was below the detection limit in the treated and untreated cell cultures 24 hours after crosslinking. With the separate use of riboflavin or UVA-light illumination, growth factor and interleukin secretion of keratoconus keratocytes did not change significantly compared to controls ($p>0.47$, $p>0.6$). 24 hours after crosslinking, IL-6 and IL-8 secretion also did not change significantly compared to untreated control KC keratocyte cultures ($p>0.35$).

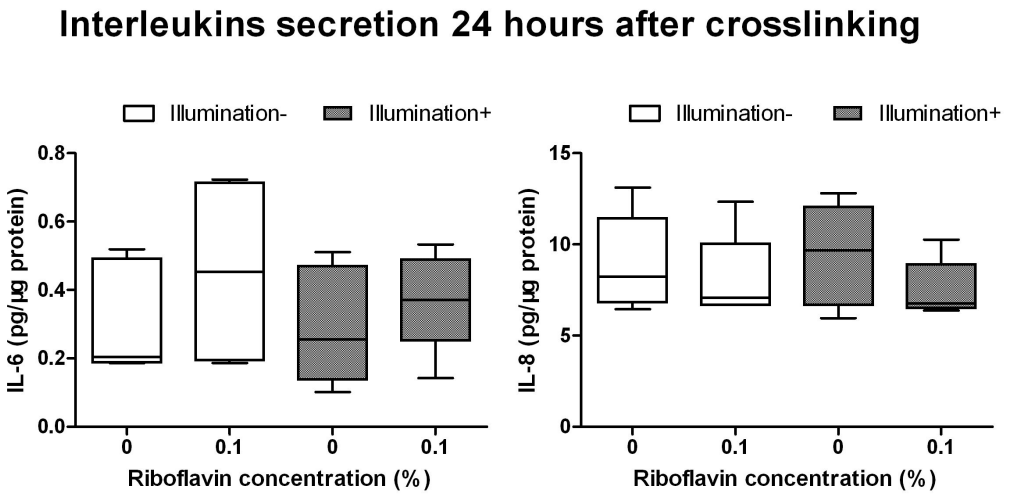

Figure 19. IL-6 and IL-8 secretion of keratocytes (mean pg/µg protein ± SD) ***24 hours*** **following crosslinking.**

	Keratocytes	Keratocytes + riboflavin	Keratocytes + 370nm	Keratocytes + riboflavin + 370nm	* p-value	** p-value	*** p-value
IL-1β	n.d.	n.d.	n.d.	n.d.	n/a	n/a	n/a
IL-6	0.31±0.16	0.45±0.26	0.29±0.17	0.37±0.15	0.53	0.6	0.75
IL-8	8.95±2.64	8.11±2.41	9.43±2.79	7.52±1.59	0.47	0.92	0.35

Table 8. Concentration (pg/µg protein) of different cytokines in the supernatant of keratoconus keratocyte cultures 24 hours after crosslinking. Values indicate mean± SD.

* p-values indicate the difference between "Keratocytes" versus "Keratocytes + Riboflavin" groups.

** p-values indicate the difference between "Keratocytes" versus "Keratocytes + 370 nm" groups.

*** p-values indicate the difference between "Keratocytes" versus "Keratocytes + Riboflavin + 370 nm" groups.

Significant values are shown in bold (Mann-Whitney U test).

n.d. = not detectable

n/a = not applicable

4 DISCUSSION

4.1 Effects of thyroxine and hydrocortisone on human keratocytes in normal and keratoconus corneas *in vitro.*

The potential clinical association between corneal ectatic disease and thyroid gland dysfunction has been known for several decades. Clinical observations of hypothyroidism in patients with keratoconus were first reported by Appelbaum in mid-1930's. [81] King subsequently reported the appearance of keratoconus following thyroidectomy. [4] Lang et al. described a case of Alagille syndrome with keratoconus and secondary hypothyroidism. [82] More recently, Kocak et al. reported a case of bilateral keratoconus associated with Hashimoto's disease. [83]

The implication of thyroid hormones in corneal embryogenesis and physiology is of paramount importance, since it is well-established that thyroid hormones play a crucial role in corneal dehydration and transparency during embryonic development. [33] Conrad et al. have reported the presence of thyroxine receptors alpha and beta in the chicken cornea, [34] while our research group has recently documented the expression of thyroxin receptors in human cornea (data under review). Moreover, thyroid hormonal changes and female sex hormones affect distinctly ocular physiology and pathophysiology during pregnancy. [84-92] This has been highlighted by recent case reports of keratoconic progression, with development of acute corneal hydrops, induced by thyroxinemia during pregnancy [3] and pregnancy-associated exacerbation of iatrogenic ectasia. [86,87]

Kahan et al. were the first to show that tear thyroxine levels in patients with keratoconus, independently of their thyroid function, were 2-50 times higher than that of normal subjects. [5] They found that tear thyroxine levels were higher during the progression of keratoconus and declined once the cornea1 curvature reached a new steady value.

At a molecular level, thyroxine functions as a transcription regulator, binding to nuclear thyroxine receptors α (THRα) and β (THRβ), which bind DNA thyroxine receptor response elements (TREs) and recruit either corepressor coactivator complexes that subsequently repress or activate target gene transcription. [93]

Conrad et al. documented that all corneal layers of the chick cornea express THRA mRNA, while keratocytes and endothelial cells alone express THRB mRNA. [34] During normal development, THRB expression increases 20-fold from embryonic day 12 to embryonic day 20, whereas THRA expression remains constant. [34] Exposure of chicken embryonic corneas to thyroxine in vitro affected significantly the expression of corneal transparency-associated genes, which control the keratan sulfate proteoglycan (KSPG) synthesis and the production of crystallins. [33]

Xie et al. demonstrated that the functional expression of Toll-like receptors-2 and -4 in human corneal epithelial cells is enhanced *in vitro* by low-concentration hydrocortisone. [94] Toll-like receptors (TLRs) play an essential role in triggering the innate immune response by recognizing pathogen-associated molecular patterns and stimulating the activity of host immune cells against several microbial products. [95] It has been well established that TLR are expressed on human corneal epithelium and play an important role in cornea protection against microbial infection. [96]

In our study, we investigated the effect of thyroxine and hydrocortisone on normal and keratoconus human corneal keratocytes *in vitro*. Exposure to thyroxine significantly increased the proliferation of normal keratocytes. Proliferation of keratoconus keratocytes only increased after treatment with very high thyroxine concentrations.

Interestingly, we discovered that TGFβ1 and FGFb, which stimulate the proliferation of NHPK, induced decreased proliferation of KHPK. Ley et al. reported that FGFb acts in autocrine and paracrine manner to stimulate corneal fibroplasias, [97] while Maier et al. showed that active TGFβ is increased in the aqueous humor of keratoconus patients. [98] Considering the effect of TGFβ1 and FGFb on KHPK in vitro, we assume that KHPK may have reduced proliferation potential compared to NHPK due to the altered corneal homeostasis in keratoconus, which may lead to expression of abnormal receptors for thyroxine, TGFβ1 and FGFb by the keratoconus keratocytes. These abnormal receptors may be inactive or present different features compared to those expressed by normal keratocytes. However, further research is required in order to confirm this working hypothesis.

Treatment with hydrocortisone *in vitro* did not significantly affect the cell proliferation of both normal and keratoconus keratocytes. Neither of the two hormones induced any significant changes in cell viability and/or cell apoptosis in normal or keratoconus keratocytes. These results suggest that thyroxine may play an important role in the cell metabolism of human corneal keratocytes in the normal

cornea, thereby explaining to some extent the reported corneal biomechanical variations induced by thyroid hormone changes. Keratoconus keratocytes appear to have reduced proliferation potential and this finding could be associated with the altered corneal homeostasis in keratoconus. Further studies are required in order to delineate the complex mechanisms of hormonal influences on corneal cell physiology and homeostasis, especially in keratoconus.

4.2 Growth factor and interleukin secretion by human normal keratocytes and keratoconus keratocytes after hormone treatment *in vitro*

Although keratoconus has primarily been described as a non-inflammatory disease, there is increasing evidence that immunological and inflammatory processes are playing a role in its pathophysiology. [6-8] The association between keratoconus and allergy, [99,100] as well as the role played by eye rubbing in the development of corneal ectatic disease, is well established. [101,102] Eye rubbing may contribute to the development of keratoconus by activating inflammatory mediators, more so than by the physical pressure applied to the eyeball. [103] Indeed, IL-1 has been implicated as a mediator of keratoconus in eye patients presenting excessive eye rubbing. [7] Moreover, patients with keratoconus demonstrate increased levels of inflammatory molecules in the tear film and the concentration of these agents has been associated with the severity of keratoconus. [8,104]

Keratocytes are the major cell type of the corneal stroma, and they contribute to the local inflammatory response by releasing various chemokines, such IL-1β, L-6, and IL-8. [105] IL-1β plays a crucial role in corneal wound healing and it is able to induce the expression of keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) by corneal keratocytes, thereby regulating the proliferation, motility, and differentiation of corneal epithelial cells following corneal surface injury. [106] IL-6 and IL-8 are important mediators of inflammatory cell response in the cornea, and are secreted in response to inflammatory stimulus. [107] IL-6 secretion by corneal keratocytes is induced following trauma or infection by IL-1α and TNFα, secreted from corneal epithelial cells. [108] IL-8 is a pro-inflammatory cytokine, produced by corneal keratocytes, which plays an important role in inflammation and wound

healing. [109,110] IL-8 has the capacity to recruit T-cells as well as nonspecific inflammatory cells into sites of inflammation by activating neutrophils. [111] Furthermore, IL-8 is chemotactic for fibroblasts and accelerates their migration and can stimulate deposition of tenascin, fibronectin, and collagen I during wound healing in vivo. [112] Both human corneal keratocytes and epithelial cells have been shown to synthesize and release IL-8 following cytokine stimulation and/or infection. [113,114]

On the other hand, fibroblast growth factor basic (FGFb), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and transforming growth factor-β1 (TGFβ1) represent important mediators of corneal stromal-epithelial interactions and they are involved in corneal wound healing. [115-118] Furthermore, TGFβ1 is involved in inflammatory reaction and induces myofibroblast differentiation, while FGFb promotes angiogenesis, cell proliferation and migration, inducing the differentiation of keratocytes into a fibroblastic phenotype. [119] HGF delays the process of re-epithelialization, induces myofibroblastic transformation of keratocytes, while it also has an inhibitory effect on cell apoptosis. [120,121]

The aim of this study was to evaluate the effect of thyroxine and hydrocortisone on the secretion of growth factors and interleukins by normal human keratocytes and keratoconus keratocytes *in vitro.* It is has been reported that all corneal layers in chicken cornea express thyroxine receptor alpha (TRA) mRNA, while keratocytes and endothelial cells express thyroxine receptors beta (TRB) mRNA. [111] During normal development TRA expression increases 20-fold from embryonic day 12 to embryonic day 20, whereas TRB expression remains stable. [122] Interestingly, treatment with thyroxine modifies the synthesis of keratan sulfate proteoglycan and modulates the expression of carbonic anhydrase genes in chicken cornea. [122] Our research group discovered that normal human keratocytes also express thyroxine receptors, which demonstrate significant alteration in their expression in keratoconus (data under review). Moreover, it has been documented that hydrocortisone down-regulates the expression of Toll-like receptor-2 and -4 by corneal keratocytes *in vitro*, [46] thereby modulating the innate corneal immune system, since Toll-like receptors stimulate the pro-inflammatory cytokines and chemokines, such as TNF-α, IL-6, IL-8, IL-18, and monocyte chemotactic protein-1. [47,48] The release of IL-6 and IL-8 is inhibited by hydrocortisone *in vitro*. [49]

In our study, we have shown that treatment with thyroxine significantly

downregulates the secretion of IL-6 by normal keratocytes *in vitro,* but it upregulates the secretion of IL-8 and TNFa by keratoconus keratocytes *in vitro*. These results suggest that thyroxine inhibits the inflammatory response in normal cornea *in vitro*, while it promotes the inflammation process in keratoconus cornea *in vitro*. We have recently discovered that thyroxine stimulates the proliferation of normal human keratocytes *in vitro*, while the proliferation potential of keratoconus keratocytes is significantly reduced (data under review). We have assumed that modified expression of the corneal thyroxine receptors occurring in keratoconus (data under review) may account for the observed differences in keratocyte proliferation. Differentiated expression of thyroxine receptors by corneal keratocytes as well as alteration in corneal homeostasis may also contribute to the different effect of thyroxine on interleukins in normal and keratoconus cornea *in vitro*.

On the other hand, treatment with hydrocortisone significantly down-regulates the secretion of IL-6, IL-8, FGFb, EGF and TGFβ1 by normal keratocytes *in vitro*, while it also down-regulates the secretion of IL-6, IL-8, TNFa and TGFβ1 by keratoconus keratocytes *in vitro.* These results are in agreement with other studies reporting that hydrocortisone inhibits the release of IL-6 and IL-8, decreasing the impact of the inflammatory cascade. [47-49] Moreover, we observed that hydrocortisone down-regulates the production of growth factors, which induce angiogenesis and differentiation of keratocytes into myofibroblasts, thereby playing a significant role in corneal wound healing.

Our results suggest that hormonal influences may modulate the secretion of interleukins and growth factors by human corneal keratocytes *in vitro,* thereby affecting the inflammatory response in corneal stroma. The effects are different in normal and keratoconus keratocytes, which may reflect the alteration of receptors expression by corneal keratocytes in keratoconus. Further studies are required in order to delineate the pathophysiological mechanisms underlying the hormonal modulation of inflammatory response mediated by human corneal keratocytes.

4.3 Viability, apoptosis, proliferation and activation of human keratoconus keratocytes after crosslinking/ riboflavin-UVA-photodynamic therapy (PDT)

Corneal collagen crosslinking was first introduced by Wollensak, Spoerl and Seiler to inhibit or stop disease progression and to increase the biomechanical stability of the cornea in KC. [9] It has been documented that crosslinking flattens keratometric readings, reduces cone progression and even improves best corrected visual acuity. [123,124]

Although crosslinking is in clinical use, to the best of our knowledge, the impact of crosslinking on KC keratocytes has not yet been analysed in detail.

The present study showed that in human KC keratocytes viability decreases, apoptosis is triggered and proliferation is inhibited, however multipotent haematopoietic stem cell transformation and myofibroblastic transformation remains unchanged 24 hours after treatment.

Viability and apoptosis of normal keratocytes have been analysed by several authors following crosslining in the past: Wollensak et al. have shown an abrupt cytotoxic effect *in vitro* on normal porcine keratocytes and Grobe et al. detected significantly decreased cell viability in a human keratocyte cell line. [126,127] Recently, our research group has shown that crosslinking decreases viability, however this does not have an impact on apoptosis of primary normal human keratocytes *in vitro*. Interestingly, in primary human KC keratocytes our present study revealed the triggering of apoptosis 24 hours after crosslinking treatment.

Kim et al. and Kaldawy et al. described that an increased percentage of apoptotic keratocytes is present in human KC corneas compared to normal human controls. [128] Macé et al. suggested that cell loss resulting from antiproliferative and hyperapoptotic phenotypes may be responsible for the pathogenesis of KC. [129] In addition, changes in corneal protein pattern, increase in enzymatic activities and cell apoptosis are also thought to be part of KC progression. [130] Chwa et al. described an increased basal generation of reactive oxygen species and reactive nitrogen species in KC keratocytes.[131]

Five to 30 months following crosslinking therapy, Messmer et al. described keratocyte damage, increased antiapoptitic bax and/or antiapoptotic surviving protein expression in keratocytes compared to untreated KC controls. [132] In our opinion, the programmed cell death of keratocytes has to be further analysed at the cellular-subcellular level, alterations of the apoptotic pathways in normal and KC keratocytes have to be

described (also following crosslinking), so that we are better able to understand the disease.

The decreased proliferation of KC keratocytes 24 hours after crosslinking in our present study is not in accordance with the published *in vivo* results six months after treatment, which have shown an increased proliferation (Ki67 positivity) of the cells at this time point. [133] In our opinion the triggered proliferation at six months after CXL is part of the late wound healing response and proliferation after 24 hours is likely to be also inhibited *in vivo*. Our previous study also reported on decreased proliferation of primary human keratocytes 24 hours following chlorine e6 photodynamic therapy in accordance with our present riboflavin-PDT results. [134]

CD34 positive keratocytes are confirmed to be multipotent haemopoetic stem cells which may play a role in cytoadhesion and signaling related to differentiation and proliferation. [135-137] Meanwhile, α-SMA is agreed to be a marker of myofibroblasts. Corneal myofibroblasts synthesize and secrete collagen I and play an important role in wound healing and contraction. [136] Furthermore, recent studies revealed that by expressing toll-like receptor (TLR), corneal myofibroblasts may take part in pathogen clearance. [136] It is also suggested that myofibroblast differentiation down-regulates keratocyte CD34 expression. [138]

Several authors reported on the occurrence of early bacterial or acanthamoeba keratitis after corneal crosslinking. Our previous study found induced myofibroblastic transformation and multipotent haematopoietic stem cell transformation in normal human keratocytes 24 hours after CXL. [139] These results do not support the hypothesis that CXL suppresses the inflammatory response of keratocytes, which leads to an increased incidence of infectious keratitis after CXL. In contrast, the inflammatory response should be promoted through CXL.

Most interestingly, in human KC keratocytes, CD34 and α-SMA expression remained unchanged following CXL. In other words, the percentage of multipotent haemopoetic stem cells and myofibroblasts remained unchanged after treatment, compared to untreated KC keratocytes. Therefore, we also do not see an increased risk of infectious keratitis in KC patients after crosslinking. At least this should not be correlated with intraoperative modification of the cellular response.

In summary, crosslinking/riboflavin-UVA-photodynamic-inactivation decreases viability, triggers apoptosis and inhibits proliferation, however it does not have an impact on multipotent haematopoietic stem cell transformation and myofibroblastic

transformation of human KC keratocytes *in vitro*. We oppose the idea that crosslinking may induce bacterial or acanthamoeba keratitis. Alterations of intracellular pathways following crosslinking have to be further analysed in the future.

4.4 Growth factor and interleukin secretion of human keratoconus keratocytes after crosslinking / riboflavin-UVA-photodynamic treatment (PDT)

The present study determined that CXL triggers FGFb secretion of keratocytes transiently (5 h). In the short term, CXL does not have an impact on HGF, TGFβ1, VEGF, KGF, IL-1β, IL-6, and IL-8 secretion of KC keratocytes, in vitro.

FGF promotes angiogenesis, cell proliferation and migration and also induces the differentiation of keratocytes into a fibroblastic phenotype. [141] In accordance with that there was no impact of CXL on myofibroblastic transformation and multipotent haemopoetic stem cell transformation of keratoconus keratocytes in our recent study. In contrast, with decreased FGFb secretion after CXL in normal keratocytes, both (normal and KC) keratocyte forms were activated after treatment. Ley at al. described that ultraviolet light induces FGFb, due to DNA damage. [97] In accordance with that we saw an increased FGFb secretion after CXL and also induced apoptosis of KC keratocytes in a former study. In addition, FGFb secretion was decreased in normal keratocytes 5 and 24 h following CXL and we could not detect triggered apoptosis of these cells following CXL. [142,143]

It is known that HGF and TGFβ1 induce myofibroblastic transformation of keratocytes. Our present results with stable HGF and TGFβ1 secretion of the cells 5 and 24 h after treatment also were in accordance with results of our previous studies: there was no significant impact of CXL on myofibroblastic transformation or multipotent haemopoetic stem cell transformation of keratoconus keratocytes. [142,143]

HGF and KGF are secreted through the stromal cells of the cornea and are known to inhibit the process of epithelialization. [144-146] With unchanged HGF and KGF secretion of keratoconus keratocytes following CXL, we do not expect an impact of CXL on corneal epithelial proliferation or migration. Interestingly, in our previous work on normal keratocytes after CXL, both HGF and KGF were down-regulated. The response of different keratocyte cell types on CXL should be further analysed. [142,143]

Lee et al. reported on the inhibitory effect of HGF on apoptosis of the cells. We demonstrated that in normal keratocytes higher concentration of HGF (compared to our present study) after CXL prevented keratocytes from apoptosis. [149] In contrast, in our present study, a relative lower HGF secretion of KC keratocytes might be related to triggered apoptosis. [142,143]

Interestingly, in our recent study, 5 h after CXL of normal keratocytes, VEGF secretion was significantly decreased, which indicates that in normal cells riboflavin-UVA-PDT may inhibit haemangiogenesis and lymphangiogenesis in the short term. In contrast, in our present study we verified no changes in VEGF secretion of keratoconus keratocytes following crosslinking. Therefore, CXL may not have an impact on corneal haemangiogenesis and lymphangiogenesis in keratoconus patients in the short term. [143]

Konstantopoulos, Hirano and Kick reported of IL-1β, IL-6 and IL-8 as the key cytokines in corneal inflammation. [148-152] These cytokines were shown to be chemotactic for riboflavin in infected corneal cells, which can cause corneal destruction. Previously we detected significantly increased IL-6 secretion in normal keratocytes after CXL, however in our present study on keratoconus keratocytes, IL-6 and IL-8 secretion did not change significantly after treatment. Therefore, we do not expect an impact of CXL on the inflammatory response of keratoconus patients, but we do expect a positive effect of the treatment (chemotactic for riboflavin) in non-keratoconic corneas, e.g. with infectious keratitis.

IL-1 is a key factor in the corneal wound healing cascade, which leads to keratocyte apoptosis and/or necrosis after epithelial injury and the effect appears to be mediated via the Fas/Fas ligand system. [153-155] In the present research, IL-1 was not detected in the supernatant of KC keratocytes, but it was present in normal keratocyte cultures. [144] This indicates that IL-1 does not seem to play a key role in the wound healing cascade after CXL, at least in vitro.

Oleinick et al have reported that IL-6-induced apoptosis is an important mode of photodynamic therapy induced cell death. [156] Furthermore, in a research with IL-6 transfected cells, apoptotic response was suggested to occur by induction of IL-6 expression which activates bax and bcl-2 after photodynamic therapy. [157] In our present study, IL-6 remained unchanged after PDT in KC keratocytes, however, it was down-regulated in normal keratocytes in a previous study. [142]

In keratoconus keratocytes we detected triggered apoptosis after crosslinking in a previous study and in the same time an increased apoptosis supporting FGFb and unchanged apoptosis triggering IL-1 and IL-6 secretion and unchanged apoptosis inhibiting HGF secretion. [142,157] The stimulus of cytokins on the apoptotic pathways of different keratoctye types after CXL has to be further clarified.

In conclusion, crosslinking triggers FGFb secretion of *keratoconus* keratocytes transiently (5 h), which normalizes after 24 h. Crosslinking does not seem to have an impact on HGF, TGFβ1, VEGF, KGF, IL-1β, IL-6, and IL-8 secretion of *keratoconus* keratocytes in the short term.

Growth factor and interleukin secretion of normal and keratoconus keratocytes differs after crosslinking, dependent on the response of keratocytes (activation or apoptotis) to the treatment in both conditions (normal and KC keratocytes). Alterations of the intracellular pathways after crosslinking have to be further analyzed in the future.

4.5 Conclusions and outlook to the future

In our work we showed that, keratoconus and normal keratocytes are different in proliferation potential and inflammation responding to exposure of thyroxine. Hydrocortisone decreases the inflammatory cascade of both cell types. Photodynamic treatment on keratoconus patients may start with short-time injury and unchanged corneal homeostasis, as well as unchanged healing potential, then make benefit on biomechanical stability after a relative long-term recovery.

Keratoconus keratocytes appear to have reduced proliferation potential and this finding could be associated with the altered corneal homeostasis in keratoconus. Further studies are required in order to delineate the complex mechanisms of hormonal influences on corneal cell physiology and homeostasis, especially in keratoconus.

Our results suggest that hormonal influences may modulate the secretion of interleukins and growth factors by human corneal keratocytes *in vitro,* thereby affecting the inflammatory response in corneal stroma. The effects are different in normal and keratoconus keratocytes, which may reflect the alteration of receptors expression by corneal keratocytes in keratoconus. Further studies are required in order to delineate the pathophysiological mechanisms underlying the hormonal modulation

of inflammatory response mediated by human corneal keratocytes.

Crosslinking/riboflavin-UVA-PDT decreases viability, triggers apoptosis and inhibits proliferation, however it does not have an impact on multipotent haematopoietic stem cell transformation and myofibroblastic transformation of human KC keratocytes *in vitro*. We oppose the idea that crosslinking may induce bacterial or acanthamoeba keratitis. Alterations of intracellular pathways following crosslinking have to be further analysed in the future.

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PUBLICATIONS

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