Department of Ophthalmology
Saarland University Medical Center, Homburg/Saar
Chairman: Prof. Dr. Berthold Seitz

Crosslinking (CXL), Autologous Serum (AS), Amniotic Membrane Suspension (AMS) and Amniotic Membrane Homogenate (AMH): Promising Tools to Improve Corneal Epithelial Wound Healing?

Dissertation for the degree of
Doctor of Medicine and Natural Sciences (MD/PhD)
Faculty of Medicine

UNIVERSITY OF SAARLAND

2016

Submitted by Ming-Feng Wu
Born on May 31, 1988 in Guangxi, P.R. China

Aus der Klinik für Augenheilkunde
Universitätsklinikum des Saarlandes (UKS), Homburg/Saar
Direktor: Prof. Dr. med. Berthold Seitz

Crosslinking (CXL), Autologes Serum (AS), Amnionmembran Suspension (AMS) und Amnionmembran Homogenat (AMH): Vielversprechende Werkzeuge zur Verbesserung der Wundheilung des Hornhautepithels?

Dissertation zur Erlangung des Grades eines
Doktors der Medizin und der Naturwissenschaften (MD/PhD)
der Medizinischen Fakultät

der UNIVERSITÄT DES SAARLANDES

2016

vorgelegt von Ming-Feng Wu
geb. am 31. Mai 1988 in Guangxi, V.R. China

Tag der mündlichen Prüfung:

Dekan :

1. Berichterstatter:

2. Berichterstatter:

3. Berichterstatter:

To my parents

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, 31.5.2016

Ming-Feng Wu

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AM	Amniotic membrane
AMH	Amniotic membrane homogenate
AMS	Amniotic membrane suspension
AME	Amniotic membrane extract
AS	Autologous serum
BSS	Balanced salt solution
FGFb	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine
CEC	Corneal epithelial cell
CXL	Crosslinking
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
DMEM/F12	Dulbecco's modified eagle medium: nutrient mixture F-12
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GEE	Generalized estimating equations
HGF	Hepatocyte growth factor
HCEC	Human corneal epithelial cell
hEGF	Human epidermal growth factor
HLA	Human leukocyte antigen
IGF	Insulin-like growth factor
IL-1	Interleukin-1
KGF	Keratinocyte growth factor
KCS	Keratoconjunctivitis sicca
mRNA	Messenger ribonucleic acid
NGF	Nerve growth factor
P/S	Penicillin-streptomycin
PBS	Phosphate-buffered saline
PEDF	Pigment epithelium-derived factor
PDGF	Platelet-derived growth factor

SD	Standard deviation
TGF-α	Transforming growth factor α
TGF-β	Transforming growth factor β
TGF-β1	Transforming growth factor β1
TGF-β2	Transforming growth factor β2
UVA	Ultraviolet A
VEGF	Vascular endothelial growth factor

SUMMARY

Crosslinking (CXL), Autologous Serum (AS), Amniotic Membrane Suspension (AMS) and Amniotic Membrane Homogenate (AMH): Promising Tools to Improve Corneal Wound Healing?

Background and Purposes: Migration and proliferation of corneal epithelial cells are one of the most fundamental processes during corneal wound healing. Corneal limbal stem cells keep proliferating, differentiating and centripetally migrating to renew the epithelium. CXL, AS and amniotic membrane extract have been reported to support corneal epithelialization and wound healing and contain several growth factors.

The *purposes* of our studies were:

- To evaluate the effect of keratocyte supernatant after CXL (harvesting time, riboflavin concentration and UVA-light illumination) on migration and proliferation of human corneal epithelial cells (HCECs), in vitro.
- To study and compare the dose-dependent effects of AS and FBS on HCEC migration, proliferation and viability in vitro, and to determine the concentrations and effects of KGF, FGFb, HGF and TGF-β1 in AS.
- To analyze the effects of different concentrations of AMS and AMH on HCEC viability, migration and proliferation in vitro, and to determine the concentrations and the influence of KGF, FGFb, HGF and TGF-β1 concentration in AMS and AMH.

Methods:

- Primary human keratocytes isolated from 8 normal and 6 keratoconus corneas were cultured. Thereafter, keratocytes in 0%, 0.05% or 1% riboflavin solution were split into samples without and with 370 nm UVA-light-illumination. After removal of the riboflavin solution, keratocytes were incubated in the mentioned keratocyte culture medium at 37 °C and keratocyte supernatant was harvested after 5 and 24 hours, respectively. Keratocyte supernatant without riboflavin and UVA treatment, was used as control. HCECs were cultured in DMEM/F12 with 5% FBS, 0.5%

DMSO, 10 ng/mL human epidermal growth factor, 1% insulin-transferrin-selenium, until reaching confluence, the HCEC culture medium was replaced by the keratocyte supernatant and HCEC migration was analyzed using wound healing assay. HCEC proliferation was determined by the cell proliferation ELISA BrdU (colorimetric) kit. Statistical analysis was performed using a linear mixed model in the framework of a Generalized Estimating Equations (GEE) approach to analyze the effect of harvesting time, riboflavin concentration and UVA-light illumination.

- AS was prepared from 13 patients according to the regulations of the LIONS Cornea Bank Saar-Lor-Lux, Trier/Westpfalz. HCECs were firstly cultured as described above, then were incubated in serum media which was consisting of DMEM/F12 supplemented by 5%, 10%, 15% or 30% AS or FBS for 24 hours. Thereafter, HCEC viability was analyzed using Cell Proliferation Kit XTT, HCEC migration and proliferation was analyzed as described above. KGF, FGFb, HGF and TGF-β1 in AS was measured by ELISA. Statistical analysis was performed using generalized linear models to analyze the effect of AS and FBS, and to analyze the responses of HCEC viability, migration and proliferation to concentrations of KGF, FGFb, HGF, TGF-β1 in AS.
- Amniotic membranes of 13 placentas were prepared and thereafter stored at -80°C using the standard methods of the LIONS Cornea Bank Saar-Lor-Lux, Trier/Westpfalz. For AMS preparation, following defreezing, AM pieces were inserted in a 6-well plate and 5 ml DMEM/F12 (with 5% FBS) per gram tissue was added for 96 hours of incubation. After removal of the amniotic membrane, the remaining supernatant was collected for experiments. For AMH preparation, following defreezing, amniotic membranes were first homogenized in liquid nitrogen. After liquid nitrogen evaporated, 5 ml DMEM/F12 (with 5% FBS) per gram tissue was added. Following centrifugation (1000 rpm for 5 min), the supernatant was collected for experiments. HCECs were firstly cultured as described above, then were incubated in culture medium using DMEM/F12, 5% FBS supplemented by 15%, 30% or 100% AMS or 15% or 30% AMH. Thereafter, HCEC viability, migration and proliferation were analyzed as described above. KGF, FGFb, HGF and TGF-β1 in AS and AMH were measured by ELISA. Comparison to the control group was performed using Mann-Whitney U test, generalized linear model was used to analyze the responses of HCEC viability, migration and

proliferation to concentrations of KGF, FGFb, HGF, TGF-β1.

Results

- Riboflavin concentration, UVA-light illumination and harvesting time of normal or keratoconus keratocyte supernatant had no significant impact on HCEC proliferation ($P > 0.10$). Riboflavin concentration did no show significant impact on HCEC migration using normal or keratoconus keratocyte supernatant ($P > 0.10$). However, longer harvesting time of normal or keratoconus keratocyte supernatant significantly increased ($P = 0.01$ for both) and UVA-light illumination of keratoconus keratocyte supernatant ($P < 0.001$) significantly decreased HCEC migration.
- HCEC viability was the highest at 30% AS or 15% FBS and the lowest at 10% AS or 30% FBS application. HCEC migration was the quickest through 30% AS or 30% FBS and the slowest through 5% AS or 5% FBS concentrations. Proliferation was the most increased through 15% AS or 5% FBS and the least increased through 30% AS or 30% FBS concentrations. HCEC viability at 10% and 15% AS was significantly worse ($P = 0.001$, $P = 0.023$) compared to baseline and significantly better at 15% FBS ($P = 0.003$) concentrations. HCEC migration was significantly worse ($P \leq 0.007$) and HCEC proliferation significantly better ($P < 0.001$) in all concentration groups compared to baseline.
- HCEC viability remained unchanged using 15% or 30% AMS ($P = 1.0$ for both), however, it decreased significantly using 100% AMS ($P < 0.001$) or 15% ($P = 0.041$) or 30% AMH ($P < 0.001$), compared to controls. Using 15% or 30% AMS, HCEC migration increased significantly ($P < 0.001$ for both). Using 15% or 30% AMH ($P = 0.153$; $P = 0.083$), HCEC migration remained unchanged and 100% AMS inhibited HCEC migration ($P < 0.001$). 15% and 30% AMS had no effect on HCEC proliferation ($P = 0.454$ and $P = 0.119$), but 100% AMS ($P < 0.001$) and 15% ($P = 0.002$) and 30% AMH ($P = 0.001$) inhibited HCEC proliferation significantly.

Conclusions

- Harvesting time, riboflavin concentration and UVA-light illumination of normal and keratoconus keratocyte cultures has no impact on proliferation of HCECs, in the short term. However, 24 hours harvesting time (both for normal and keratoconus keratocytes) increases and UVA-light-illumination of keratoconus

keratocyte cultures decreases HCEC migration.

- HCEC viability is mostly increased through 30% AS or 15% FBS, migration through 30% AS or 30% FBS and proliferation through 15% AS or 5% FBS. In addition, AS better supports HCECs viability and migration than FBS.
- With unchanged HCEC viability and proliferation and increased HCEC migration, 15% and 30% AMS application seems to be the most appropriate method to support epithelial healing.

ZUSAMMENFASSUNG

Crosslinking (CXL), autologes Serum (AS), Amnionmembran Suspension (AMS) und Amnionmembran Homogenat (AMH): Vielversprechende Wekzeuge zur Verbesserung der Wundheilung des Hornhautepithels?

Hintergrund / Ziele: Die Migration und Proliferation spielt eine entscheidende Rolle in der kornealen Wundheilung. Korneale limbale Stammzellen sorgen für eine ständige Erneuerung des Epithels durch Proliferation, Differenzierung und Migration. CXL, AS und Amnionmembran enthalten verschiedene Wachstumsfaktoren, die die Wundheilung des kornealen Epithels unterstützen.
Die Ziele dieser Studie waren:

- Den Effekt von Zellkulturüberständen von Keratozyten nach CXL (Abnahmezeitpunkt, Riboflavin-Konzentration und UVA-Licht Einfluss) hinsichtlich der Migration und Proliferation humaner kornealer Epithelzelllinien (HCEC) in vitro zu untersuchen.
- Der Vergleich Dosis-abhängiger Effekte von AS und FBS auf die Migration, Proliferation und Viabilität von HCEC's in vitro, sowie die Bestimmung der Konzentrationen und Effekte von KGF, FGFb, HGF und TGF-β1 im autologen Serum.
- Den Effekt unterschiedlicher Konzentrationen von AMS und AMH auf die Migration und Proliferation von HCEC's in vitro, sowie die Bestimmung der Konzentration und Effekte von KGF, FGFb, HGF und TGF-β1 Konzentration in AMS und AMH.

Methoden:

- Primäre humane Keratozyten wurden von 8 gesunden und von 6 Patienten mit diagnostiziertem Keratokonus isoliert und kultiviert. Die Keratozyten wurden mit einer Riboflavin-Lösung der Konzentrationen 0%, 0,05% und 0,1% versehen. Eine Gruppe wurde mit einer Wellenlänge von 370 nm UVA-Licht bestrahlt, eine Gruppe blieb unbestrahlt. Nach der Bestrahlung wurde die Riboflavin-Lösung entfernt und durch Kulturmedium ersetzt. Nach jeweils 5

Stunden und 24 Stunden Inkubation wurde das Kulturmedium entnommen. Als Kontrolle dienten Kulturüberstände ohne Riboflavin und ohne Bestrahlung. HCEC's wurden in DMEM/F12 supplementiert mit 5% FBS, 0,5% DMSO, 10 ng/mL humanem Epidermal Growth Factor, 1% Insulin-Transferrin-Selenium bis zur Konfluenz kultiviert. Das Medium wurde gegen die Zellkulturüberstände der Keratozytenkulturen ersetzt. Die Migration wurde mit einem Scratch-Assay analysiert, die Bestimmung der Proliferation erfolgte kolorimetrisch. Die statistische Auswertung erfolgte mit einem linearen generalisierten Modell (GEE), um die Einflussgrößen der Inkubationszeit, der Riboflavin-Konzentration und des UVA-Lichts unabhängig voneinander zu bestimmen.

- Das autologe Serum von 13 Patienten wurde nach den Richtlinien der LIONS Hornhaut Bank Saar-Lor-Lux, Trier/Westpfalz präpariert. HCEC's wurden wie beschrieben kultiviert und für 24 Stunden mit DMEM/F12 supplementiert entweder mit 5%, 10%, 15% und 30% AS oder FBS inkubiert. Die Viabilität der HCEC's wurde mit einem XTT Zellproliferations-Test Kit kolorimetrisch erfasst. Die Migration und Proliferation wurden wie bereits beschrieben analysiert. Die Konzentration von KGF, FGFb, HGF und TGF-β1 in AS wurde mit einem ELISA gemessen. Die Statistische Analyse erfolgte mit einem linearen generalisiertem Modell um den Einfluss von AS und FBS auf die Viabilität, Migration und Proliferation in Abhängigkeit von KGF, FGFb, HGF, TGF-β1 in AS zu erfassen.
- Amnionmembranen von 13 Patientinnen wurden nach den Richtlinien der LIONS Hornhautbank Saar-Lor-Lux, Trier/Westpfalz präpariert und bei -80°C tiefgefroren. Für die Präparation der AMS wurden die Membranen in kleine Stücke geschnitten und mit 5ml DMEM/F12 (+ 5% FBS) pro Gramm Gewebe für 96 Stunden inkubiert. Das Medium ohne Amnionmembran wurde für die Testansätze verwendet. Für die Präparation des AMH wurden die Membranen in flüssigem Stickstoff homogenisiert und das Homogenat in 5ml DMEM/F12 (+ 5% FBS) pro Gramm Gewebe aufgenommen. Für die Experimente wurde der Überstand des Homogenates verwendet. Die HCEC's wurden wie schon beschrieben kultiviert und mit DMEM/F12, 5% FBS und jeweils 15%, 30% oder 100% AMS, oder 15% oder 30% AMH inkubiert und die Migraton und

Proliferation bestimmt. Die Messung der Konzentrationen von KGF, FGFb, HGF and TGF-β1 in AMS und AMH erfolgte mit einem ELISA.Die Vergleiche zu den Kontrollgruppen wurden mit dem Mann-Whitney U-Test analysiert, ein lineares generalisiertes Model diente zur Erfassung der Einfluss von KGF, FGFb, HGF, TGF-β1 auf die Migration und Proliferation der HCEC's.

Ergebnisse:

- Die Riboflavin-Konzentration, UVA-Bestrahlung und Abnahmezeit des Mediums von normalen oder Keratokonus-Keratozyten zeigen keinen Einfluss auf die Proliferation der HCEC's ($P > 0,1$). Die Riboflavin-Konzentration im Medium von normalem oder Keratokonus-Keratozyten hatte keinen Einfluss auf die Migration der HCEC's ($P > 0,1$), eine spätere Abnahme-Zeit des Mediums von normalen und Keratokonus-Keratozyten erhöhte die Migrationsrate der HCEC's ($P = 0,01$). Die Bestrahlung der Keratokonus-Keratozyten mit UVA-Licht hingegen verlangsamte die Migration der HCEC's ($P < 0,001$).
- Die Viabilität der HCEC's zeigte die höchsten Werte bei dem Einsatz von 30% AS und 15% FBS, die niedrigsten Werte bei 10% AS und 30% FBS. Die Migrationsrate war bei Verwendung von 30% AS und 30% FBS am höchsten und zeigte bei 15% AS und 5% FBS die geringsten Migrationsraten. Die Proliferationsrate war bei einem Einsatz von 15% AS und 5% FBS am höchsten und zeigte unter Verwendung von 30% AS und 30% FBS die geringsten Proliferationsraten. Die Viabilität der HCEC's zeigte die schlechtesten Werte bei Verwendung von 10% und 15% AS ($P = 0,001$, $P = 0,023$) im Vergleich zur Basislinie und die besten Werte bei Einsatz von 15% FBS ($P = 0,003$). Die Migration der HCEC's zeigte in allen Gruppen signifikant schlechtere Werte ($P \leq 0,007$), die Proliferation hingegen signifikant bessere Werte ($P < 0,001$) im Vergleich zur Basislinie.
- Die Viabilität der HCEC's zeigte keine Veränderung bei Verwendung von 15% und 30% AMS (beide $P = 1,0$). Eine signifikant schlechtere Viabilität zeigt sich unter Verwendung von 100% AMS ($P < 0,001$) und 15% bzw. 30% AMH ($P < 0,001$), im Vergleich zur Kontrolle. Bei Einsatz von 15% und 30% AMS steigt die Migrationsrate der HCEC's signifikant an ($P < 0,001$ für beide). Die Migration zeigt keinen Unterschied bei Einsatz von 15% und 30% AMH ($P =$

0,153, $P = 0,083$), zeigt jedoch eine erniedrigte Migrationsrate bei Einsatz von 100% AMS ($P < 0,001$). Auf die Proliferation der HCEC's zeigt der Einsatz von 15% und 30% AMS keinen Effekt ($P = 0,454$, $P = 0,119$), wird jedoch bei Verwendung von 100% AMS ($P < 0,001$) und 15% und 30% AMH ($P = 0,002$, $P = 0,001$) signifikant inhibiert.

Zusammenfassung:

- Der Abnahmezeitpunkt des Mediums bei normalen und Keratokonus-Keratozyten, Riboflavin-konzentration und UVA-Licht haben keinen Einfluss auf die Proliferation von HCEC's. Eine Abnahme des Mediums nach 24 Stunden von normalen und Keratokonus-Keratozyten steigert jedoch die Migrationsrate, während der Einsatz von UVA-Licht die Migrationsrate der HCEC's hemmt.
- Die Viabilität der HCEC's ist am höchsten unter Verwendung von 30% AS und 15% FBS, die Migrationsrate bei Einsatz von 30% AS und 30% FBS und die Proliferation bei Verwendung von 15% AS und 5% FBS.
- Betrachtet man die unveränderte Viabilität und Proliferation der HCEC's bei erhöhter Migrationsrate, zeigt die Verwendung von 15% und 30% AMS das beste Resultat um die epitheliale Wundheilung zu unterstützen.

1 INTRODUCTION

Corneal epithelium is the outermost layer of the cornea, consisting of stratified cells with constant self-renewal. The regenerative capacity of the corneal epithelium is retained in its basal cell layer, which consists of two different cell populations: stem cells and transient amplifying cells [1, 2]. The epithelial stem cells reside in their niches, which are in the limbal corneal region called palisades of Vogt [3]. Asymmetric division of limbal stem cells give rise to transient amplifying cells, which can migrate towards the central cornea to compensate epithelial cell loss by desquamation [4, 5].

When corneal epithelial wound healing is initiated, asymmetric division of limbal stem cells will launch numerous transient amplifying cells that migrate centripetally in direction of the wound edge, followed by proliferation, differentiation, stratification and adhesion until the wound is closed [6, 7]. Cell migration and proliferation have a crucial role in corneal epithelial wound healing; these processes are regulated by growth factors and cytokines.

To facilitate corneal epithelial wound healing, many in part novel methods may be tested such as corneal crosslinking (CXL), application of autologous serum (AS) or amniotic membrane extract (AME).

1.1 Growth factors regulating corneal epithelial wound healing

1.1.1 Growth factors produced by keratocytes, supporting epithelial healing

Several growth factors and cytokines influence proliferation and migration of corneal epithelial cells. However, especially following corneal abrasion, growth factors and cytokines produced by keratocytes play a critical role, and influence epithelial cell proliferation and migration in a paracrine fashion [8].

Keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) are secreted through keratocytes and influence the epithelium [9, 10]. A corneal epithelial wound will stimulate up-regulation of HGF and KGF mRNA in keratocytes, while HGF and KGF receptor mRNA are up-regulated in the corneal epithelium [11].

In an in vitro experiment [9], KGF mRNA was shown to be expressed in rabbit keratocytes but not in corneal epithelial cells (CECs), while KGF receptor mRNA was expressed in CECs but not in keratocytes. An animal experiment [12] and in vitro experiments [9, 13] suggested that KGF enhanced CEC proliferation, accelerated corneal epithelial wound healing, but did not affect motility.

HGF at the ocular surface is mainly produced by the lacrimal gland [14], but HGF mRNA is also expressed in keratocytes, corneal endothelial cells and at a low level in CECs [10]. Previous experiments suggested that HGF promoted CEC migration, proliferation and inhibited apoptosis [15-18]. However, it was also reported that HGF delayed while KGF accelerated corneal epithelial wound healing [19].

The expression of FGFb is weak in the normal cornea, but strong at the wound edge of the corneal epithelium, stroma and endothelium [20]. In human studies, FGFb was reported to promote human corneal epithelial cell (HCEC) proliferation and accelerate wound healing and in an animal study to promote canine CEC viability [21-23].

1.1.2 TGF-β as the inhibitory factor

Three isoforms of TGF-β and their receptors are expressed in CECs and keratocytes [24]. In an uninjured mouse cornea, TGF-β1 is detected inside epithelial cells, but not

secreted, while the isoforms TGF-β2 and β3 are present in the extracellular environment. Through stimulus of a corneal wound, all three isoforms of TGF-β are secreted into the subepithelial stroma [25]. In human and equine experiments, TGF-β1 was reported to inhibit both HCEC and keratocyte proliferation [21, 26]. TGF-β1 and β2 were reported to antagonize the effects of other growth factors in vitro and inhibited rabbit CEC proliferation promoted by KGF, HGF and epidermal growth factor (EGF) [27].

In some other studies, TGF-1 was reported to promote keratocyte proliferation although delayed keratocyte migration [28, 29]. TGF-β is a crucial factor regulating fibroproliferative processes in the eye, including corneal scarring [30]. O'Kane et al. suggest that by manipulating isoforms of TGF-β (particularly by reducing the relative level of TGF-β1), corneal scarring can be reduced [31].

1.2 Photo-oxidative corneal crosslinking

1.2.1 Mechanisms of CXL

In the corneal stroma, collagen fibrils are synthesized by keratocytes and following their secretion are assembled into collagen fibres in the extracellular matrix [32]. Covalent crosslinkages between collagen fibres are necessary to maintain the stability and stiffness of the corneal stroma. Under physiological conditions, these crosslinkages between corneal collagens are produced by an enzymatic oxidation reaction catalyzed by lysyl oxidase [33].

Besides, there are also nonenzymatic methods resulting in crosslinkages between corneal collagens, including photochemical reaction or the use of chemical agents such as glutaraldehyde or aldehyde sugars [34].

During the so called crosslinking (CXL) therapy the photosensitizer riboflavin (vitamin B_2) and UVA-light illumination are used, in means of a photochemical reaction. Riboflavin is excited through UVA-light-illumination and following relaxation this photochemical reaction generates free oxygen radicals. Thereafter, amino acids along the collagen molecular chains react with each other under the effect of oxygen radicals

and covalent crosslinkages between collagens are formed [35].

1.2.2 Applications and safety of CXL

CXL was first reported to stop the progression of keratoconus in 2003 in a clinical pilot trial [36]. As it can enhance corneal stiffness, CXL is usually used in treatment of keratoconus or ectatic corneal disease [37, 38]. Clinical corneal CXL is performed as **Figure 1**.

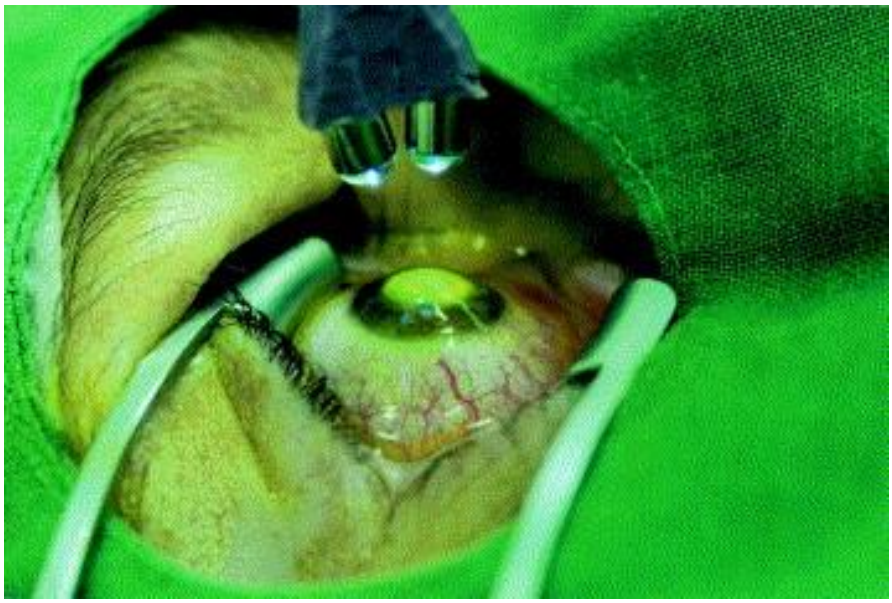

Figure 1. Corneal CXL with riboflavin and UVA-light illumination [36].

The successful application of CXL in recent years has also been reported in treatment of resistant bacterial, fungal and acanthamoeba keratitis [39-41]. In addition, CXL has been used in treatment of corneal melting and to successfully support corneal epithelialization and wound healing [42, 43].

During the CXL process, keratocytes, corneal limbal epithelial stem cells, and in transepithelial CXL the entire epithelium, may be jeopardized by the DNA damage effect of the UVA-light [44] and radicals released from riboflavin [45].

Following CXL, the density of keratocytes in the anterior stroma usually decreases significantly in vivo and it may take even 12 months for the keratocytes to recover [46-51]. Animal experiments reported that the keratocyte damage is inducible through 0.5mW/cm^2 or more UVA irradiance, which is lower than the 3mW/cm^2 irradiance used with standard CXL [52, 53].

Interestingly, most clinical studies describe corneal re-epithelialization within 3-4 days after CXL [54-61], but the corneal epithelium removed in CXL might not restore its thickness until three month [62] and apoptosis of epithelial cells following transepithelial CXL may happen [49]. The effects of CXL on CEC functions, like migration and proliferation, have not been thoroughly analyzed yet. It is only known that CXL may inhibit the regeneration of human limbal epithelial cells [63].

1.3 Autologous serum

1.3.1 Autologous serum as a natural substitute of tears

Tear film is a fluid layer essential for ocular surface lubrication, immune protection and nutrition [64]. Abnormal tear film results in keratoconjunctivitis sicca (KCS) or dry eye, which is most commonly treated by lubricating artificial tears [65]. However, the components of the tear film, including electrolytes, proteins, lipids and mucins are hardly compensated by the single use of lubricants [64].

Human peripheral blood serum is a natural substitute of tears. Serum has similar pH and osmolality to tears. Furthermore, it contains many identical components with tears, such as EGF, nerve growth factor (NGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), TGF-β, lysozyme, Ig A, albumin, vitamin A, substance P, etc. [66]. In 1984, the beneficial effect of AS, as artificial tear KCS patients was firstly reported by Fox et al [67]. Thereafter, serum eye drops, mostly autologous and sometimes allogeneic, were used in various ocular surface diseases such as KCS, Sjögren's syndrome, persistent corneal epithelial defects, chemical eye burn and neurotrophic keratitis [68-73].

1.3.2 Effects and safety of autologous serum

Like many other materials, autologous serum (AS) may only be safe and optimal to HCECs in a certain concentration range. In 2001, using an in vitro cell culture model, Geerling et al. found that 50% and 100% AS were toxic to HCECs [74]. A few years later, Liu et al. found that 100% human serum supported better migration than 25% human serum diluted with isotonic saline [75]. Later they found that the relative cell growth of HCECs was best supported with human serum diluted to 12% [76]. Besides these in vitro studies, Akyol-Salman found that 100% AS could accelerate rabbit corneal wound healing more than 20% AS [77].

Nowadays, 20% may be the most commonly used concentration of AS in both clinical and experimental settings [72, 78-82]. However, there is still no international consensus on AS preparation and application and concentration of AS can vary from 20% to 100% among different institutions [66, 83].

Recently, AS has also been recommended as an alternative of fetal bovine serum (FBS) for culturing and expansion of human corneal limbal epithelial cells in vitro for in vivo transplantation to devoid animal-derived products [84].

In some previous studies, CECs cultivated in AS and FBS supplemented media demonstrated similar morphology and expression patterns of intercellular junction proteins, basement membrane proteins and tissue-specific keratins. Likewise, BrdU ELISA cell proliferation assay and colony-forming efficiency analysis did not demonstrate significant difference between those CECs [85, 86].

Considering the complicated effects of AS on HCECs, and the inconsistency of AS application, the dose-dependent effects of this promising material, and the growth factors which may play key roles in effects of AS, should be studied in detail.

1.4 Amniotic membrane extraction

1.4.1 Amniotic membrane

Amniotic membrane (AM) is the innermost layer of fetal membranes, which contacts

the amniotic fluid like the developing fetus. AM comprises a monolayer of cuboidal epithelial cells, a thick basement membrane and an avascular stroma.

Fresh AM is rich of various growth factors, such as HGF, KGF, TGF-β, FGFb, EGF, PDGF, IGF and vascular endothelial growth factor (VEGF) etc. [87-89]. All these growth factors give AM a variety of biological characteristics, such as support of epithelialization, anti-inflammation and anti-angiogenesis [90].

The basement membrane of the AM consists of collagen, fibronectin and laminin, and it is one of the thickest membranes of the human body, with satisfactory elasticity and strength [91]. Moreover, human AM is an immune-privileged tissue despite its expression of human leukocyte antigen (HLAs). One hypothesis is that amniotic cells are apoptotic, therefore easy to disappear without causing immunologic rejection [92].

1.4.2 Novel approach in corneal application of AM

AM had been used for ocular surface reconstruction as early as 1940s [93], and gained popularity since it was reintroduced by Kim and Tseng in 1995 [94]. AM is usually transplanted to the cornea as a patch (onlay), as graft (inlay) or as multilayers (“sandwich”) [95].

However, to avoid the disadvantages of AM transplantation, like surgical contraindications, suture-related complications and cost of hospitalization, efforts had been made to seek a new form of AM therapy. By using an AM extract [96-98], or supernatant collected from an amniotic cell culture [99-101], beneficial effects were observed such as support of corneal healing, suppression of neovascularization and inflammation and amelioration of symptoms in patients [102, 103] with chemical burn, corneal ulcer, dry eye or previous keratoplasty, etc.

Guo Q et al. [104] compared a homogenate of AM to AM transplantation using a rabbit model, and found that the AMH was as effective as AM transplantation in promoting corneal healing.

In the limited number of previous studies, preparation methods of amniotic membrane extraction (AME) were not completely identical. In general, the AMs were

homogenized and centrifuged to release the containing beneficial biochemical factors. However, whether change in concentrations or preparation methods of AME could lead to different therapeutic outcome, and which biochemical factors in the AME effect the HCEC functions, have not been studied, yet.

In our present study, we prepared AME using two different methods, which we defined as amniotic membrane suspension (AMS) and amniotic membrane homogenate (AMH).

1.5 Thesis aims

Migration and proliferation of CECs are one of the most fundamental processes during corneal wound healing. The corneal limbal stem cells keep proliferating and centripetally migrating to renew the epithelium. CXL, AS, AME have been reported to support corneal epithelialization and wound healing.

The *purposes* of our studies were:

- To evaluate the effect of keratocyte supernatant after CXL (harvesting time, riboflavin concentration and UVA-light illumination) on migration and proliferation of human corneal epithelial cells (HCECs), in vitro.
- To study and compare the dose-dependent effects of AS and FBS on HCEC migration, proliferation and viability in vitro, and to determine the concentrations and effects of KGF, FGFb, HGF and TGF-β1 in AS.
- To analyze the effects of different concentrations of AMS and AMH on HCEC viability, migration and proliferation in vitro, and to determine the concentrations and effects of KGF, FGFb, HGF and TGF-β1 in AMS and AMH.

2 MATERIALS AND METHODS

2.1 Reagents

DMEM/F12 (Life technologies, Paisley, UK), fetal bovine serum (FBS) (Life technologies, Paisley, UK), penicillin-streptomycin (P/S) (Sigma-Aldrich, USA), dimethylsulfoxide (DMSO) (Central Chemical Storage of Saarland University, Saarbruecken, Germany), human epidermal growth factor (hEGF) (Biochrom, Berlin, Germany), insulin-transferrin-selenium (Life technologies, Paisley, UK), trypsin-EDTA solution (Sigma-Aldrich, USA), collagenase A (Roche, Mannheim, Germany), riboflavin 5'-phosphate sodium salt hydrate (Sigma-Aldrich, China), dextran (Sigma-Aldrich, Denmark), Culture Medium I (Biochrom GmbH, Berlin, Germany), BSS (Pharmacy of Saarland University Medical Center), benzylpenicillin (InfectoPharm, Heppenheim, Germany), streptomycin (X-GEN Pharmaceuticals, New York, USA), Cysto-Myacyne N (NEWBON, Berlin, Germany), amphotericin B (Bristol-Myers Squibb, München), glycerin (Pharmacy of Saarland University Medical Center), cell proliferation ELISA BrdU (colorimetric) kit (Roche, Mannheim, Germany), sulfuric acid (Titrisol, Darmstadt, Germany), phosphate-buffered saline (PBS) (Sigma-Aldrich, Steinheim, Germany), Cell Proliferation Kit XTT (AppliChem, Darmstadt, Germany), Human KGF/FGF-7 DuoSet (R & D systems, Minneapolis, USA), Human FGF basic DuoSet (R & D systems, Minneapolis, USA), Human HGF DuoSet (R & D systems, Minneapolis, USA), Human TGF-beta 1 DuoSet (R & D systems, Minneapolis, USA).

2.2 Isolation of keratocytes

Eight human corneas (78 ± 12 years, 62.5% male) were obtained from the LIONS Cornea Bank Saar-Lor-Lux Trier/Westpfalz (these donor corneas were not suitable for transplantation because of low endothelial cell count) and 6 keratoconus corneas (26 ± 4 years, 50% male) were obtained from keratoconus patients from planned penetrating keratoplasties.

Keratocytes were isolated as described previously [105]. In short, the human corneoscleral buttons from the Cornea Bank or pieces from the explanted keratoconus

corneal buttons were aseptically rinsed in PBS before removal of the endothelium including Descemet's membrane by sterile surgical disposable scalpel. In case of Cornea Bank corneas a central corneal button with epithelium was cut using a 8.0 mm Barron's trephine. Thereafter, donor and keratoconus corneal button pieces were 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 surgical disposable scalpel. The remaining corneal stroma was incubated in culture medium with 1.0 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 1.0 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 2 to 5 of cells was used for experiments.

2.3 Keratocyte culture

Cultured keratocytes showed typical morphologic characteristics of corneal stromal cells. After the proliferation period had started, a confluent monolayer was generally reached between day 3 and 5. Keratocytes were used at this stage for further experiments.

2.4 CXL/ Riboflavin-UVA photodynamic inactivation

Human keratocytes were seeded in 6-well tissue culture plates and were allowed to grow for 48 hours before photodynamic treatment. Cells were washed three times with PBS once before riboflavin-5-phosphate was added. The concentration of riboflavin-5-phosphate was 0%, 0.05% and 0.1% and it was diluted in 20% Dextran-PBS. Thereafter, the cells were exposed directly to UVA light (375 nm) for 4 minutes 10 seconds (2 J/cm^2). Following illumination, the riboflavin-5-phosphate solution was removed, cells were washed three times with PBS, fed with 4ml/well culture medium and cultivated at 37 °C for 5 hours or 24 hours before removal of 2 ml/well supernatant. The supernatant

was collected and stored until epithelial cell experiments at -20°C (maximal for 6 weeks).

In summary, the cells were treated with the following combinations: 0.05% and 0.1% riboflavin-5-phosphate-UVA, 0.05% and 0.1% riboflavin-5-phosphate only, UVA only. The control cells were incubated only in the dark for 4 minutes 10 seconds.

2.5 Preparation of autologous serum

AS was obtained from 13 patients (5 females, 69±16 (41 to 92) years) with the diagnosis corneal ulcer (6 patients), corneal erosion (4 patients), Salzmann's nodular degeneration (1 patient), Sjögren`s syndrome (1 patient), systemic lupus erythematosus (1 patient). 5 of the patients had previous penetrating keratoplasty. Serological tests for hepatitis B, hepatitis C, HIV, cytomegalovirus and syphilis were all negative. To prepare the AS, peripheral blood was obtained by vein puncture, was stored for 1 to 3 hours at room temperature, then centrifuged at 3000 rpm (855 g) for 15 minutes. Thereafter, under laminar flow, serum was pipetted into a sterile container and 1.5 to 2 ml aliquots of serum were filtered and injected into 5 ml sterile dropper bottles via a disposable filter connected to a syringe. The serum was stored at -20 °C for maximal 3 months.

2.6 Preparation of amniotic membrane suspension and amniotic membrane homogenate

Thirteen placentas were obtained from cesarean deliveries of 20 to 40 years old females after informed consent. Serological tests for hepatitis B, hepatitis C, HIV, cytomegalovirus and syphilis were all negative. Amniotic membranes were prepared using the standard methods of the LIONS Cornea Bank Saar-Lor-Lux, Trier/Westpfalz. Briefly, under laminar flow, amniotic membranes were bluntly separated from chorion, rinsed in BSS containing 0.05mg/ml benzylpenicillin, 0.05 mg/ml streptomycin and 0.01 mg/ml amphotericin B, were placed epithelial side up on nylon membranes, were divided into 3 × 3 cm^2 slices and then cryopreserved at -80 °C in Culture Medium I containing 0.1 mg/ml Cysto-Myacyne N, 0.05 mg/ml benzylpenicillin, 0.05 mg/ml streptomycin, 0.01 mg/ml amphotericin B and 50% glycerin.

For AMS preparation, following defreezing, amniotic membrane pieces were inserted

in 6-well plates and 5 ml DMEM/F12 (with 5% FBS) per gram tissue was added for 96 hours incubation at 37 °C. After removal of the amniotic membrane, the remaining supernatant was collected for experiments.

For AMH preparation, following defreezing, amniotic membranes were first homogenized in liquid nitrogen. After evaporation of liquid nitrogen, 5 ml DMEM/F12 (with 5% FBS) per gram tissue was added. Following centrifugation (1000 rpm for 5 min), the supernatant was collected for experiments.

2.7 Culture of human corneal epithelial cells

SV40-Adeno vector transformed human corneal epithelial cells (Cell No. RCB2280) were obtained from RIKEN BioResource Center, Ibaraki, Japan. For the following experiments, HCECs were first expanded using DMEM/F12 culture medium with 5% FBS, 5 μg/ml Insulin, 10 ng/ml hEGF and 0.5% DMSO.

2.8 Wound healing assay of human corneal epithelial cells

HCEC migration was determined by the wound healing assay. HCECs were seeded in 6-well plates and were allowed to grow until reaching confluence. Thereafter, culture medium was removed, cells were rinsed twice with PBS and HCECs were incubated at 37°C for 20 minutes with

1. the collected *keratocyte supernatant*; each HCEC culture was given a supernatant of a separate keratocyte culture;

Or 2. "*serum media*", which again consisted of DMEM/F12 supplemented by 5%, 10%, 15%, 30% AS or FBS;

Or 3. the above described *AMS or AMH containing media*.

Then, HCEC monolayers were scratched by 200 μl yellow pipette tips (Eppendorf AG, Hamburg, Germany). Thereafter, 3 to 5 images of each scratch wound were taken at the beginning and after 9 hours incubation **(Figure 2)**.

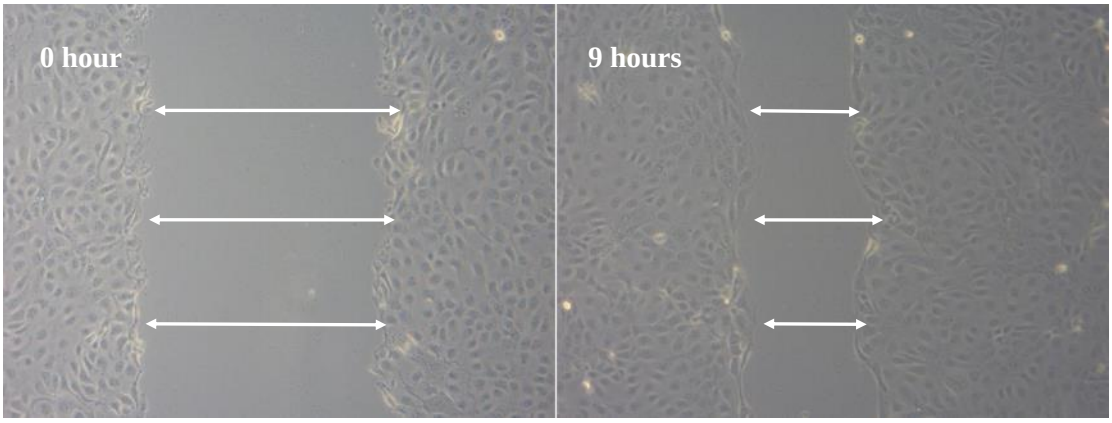

Figure 2. HCEC monolayers were scratched by 200 µl yellow pipette tips. Thereafter, 3 to 5 images of each scratched wound were taken at the beginning and after 9 hours of incubation.

As a first step, the areas of the scratched wound areas on the images were measured by the GNU Image Manipulation Program in pixels. Then, the sum of the pixel width was converted into average wound width in micrometers for each image and each time-point. At last, an average wound width was calculated for each scratch wound from the 5 images taken at one time-point. Thereafter, we analyzed statistically the width of the scratched wound for all epithelial cell cultures for each time-point.

2.9 Determination of human corneal epithelial cell proliferation

HCEC proliferation was determined using the cell proliferation ELISA-BrdU kit. This measurement analyzed BrdU incorporation into the newly synthesized cellular DNA of HCECs. The test was performed according to the manufacturer's protocol.

1. To evaluate the effect of *keratocyte supernatant* after CXL, HCECs were first suspended in standard keratocyte culture medium (consisting of DMEM/F12, 10% FBS and 1% P/S);

Or 2. To study the *effect of AS*, HCECs were first suspended in growth factor-free medium (consisting of DMEM/F12, 5% FBS and 1%P/S) to avoid the effect of growth factors;

Or 3. To study the *effects of AMS and AMH*, HCECs were also first suspended in growth factor-free medium.

Thereafter HCECs were seeded at 3×10^3 (for CXL or AS experiments) or 9×10^3 (for

AMS and AMH experiments) HCECs/cm^2 (100µL/96-well) in 96-well plates and were cultured for 24 hours until adherence.

Then, the medium was replaced by

1. the earlier (following CXL) collected *keratocyte supernatant*. Each HCEC culture was given a supernatant of a separate keratocyte culture;

Or 2. the "*serum media*";

Or 3. the above described *AMS or AMH containing media*.

After culturing the HCECs for 24 hours, 10 µl/well BrdU labeling solution was added and the tissue plates were incubated at 37 °C for 3 h (BrdU incorporation). After removal of the culture medium, the cells were fixed with FixDenat (provided with the test kit) followed by incubation with anti-BrdU-POD (monoclonal antibody to the thymidine-analogue 5-bromo-2'-deoxyuridine Fab fragments with peroxidase conjugated) (100 µl/well) for 90 minutes, which binds the incorporated DNA. Following removal of the solution, HCEC-96-well-plates were rinsed 3 times with PBS and 100 µl/well tetramethyl-benzidine substrate solution was added until color development was sufficient for photometric detection (10-30 minutes). Thereafter, 25 µl/well 1-N-sulfuric acid was added and mixed thoroughly by shaking for 1 minute. Thereafter, the 96-well plates were measured using a 96-well microplate reader (Tecan Infinite Reader, TECAN Deutschland GmbH, Crailsheim, Germany) at 450 nm (reference wavelength: 690 nm).

2.10 Determination of human corneal epithelial cell viability

HCEC viability was determined using the Cell Proliferation Kit XTT. This measurement was based on the activity of mitochondria in the living cells to change the tetrazolium salt XTT to the orange colored compounds of formazan. The test was performed according to the manufacturer's protocol.

In short, to avoid the effect of growth factors in the culture medium, HCECs were first suspended in growth factor-free medium (consisting of DMEM/F12, 5% FBS and 1%P/S). Thereafter, HCECs were seeded at 9×10^3 cells/cm^2 (100µL/96-well) in 96-well plates and were cultured for 24 hours until adherence. Then, the medium was replaced by

1. the 5, 10, 15 or 30% AS or FBS containing medium, called later in the text "*serum media*".

Or 2. the *AMS or AMH containing media* which were consisting of DMEM/F12, 5% FBS supplemented by 15%, 30% or 100% AMS or 15% or 30% AMH.
After culturing the HCECs for 24 hours, 50 µl/ well XTT-containing reaction solution was added and the cells in 96-well plates were incubated at 37 °C for 2 h. Thereafter, the 96-well plates were measured immediately using a 96-well microplate reader (Tecan Infinite Reader, TECAN Deutschland GmbH, Crailsheim, Germany) at 450 nm wavelength (reference wavelength: 690 nm).

2.11 Measurement of KGF, FGFb, HGF and TGF-β1 in autologous serum, amniotic membrane suspension and amniotic membrane homogenate

KGF, FGFb, HGF and TGF-β1 concentrations in AS, or in 100% AMS in 2 of the used amnion donors and in 100% AMH in 6 of the used donors, were measured by taking a 100 µl aliquot.
The measurements were performed following the manufactures' ELISA-protocols. The growth factor concentrations were quantified by using a human recombinant KGF, FGFb, HGF and TGF-β1 as standard. The absorbance was measured at 450 nm (Tecan Infinite Reader, TECAN Deutschland GmbH, Crailsheim, Germany). Measurement ranges were the following: KGF: 16–2000 pg/ml, FGFb: 8–1000 pg/ml, HGF: 60–8000 pg/ml and TGF-β1: 31–2000 pg/ml. Measured concentrations below the above values were considered as zero.

2.12 Statistical analysis

Statistical analysis was performed using the SPSS Statistics 22.0. $P < 0.05$ was considered statistically significant.

For statistical analysis we used a linear mixed model in the framework of *Generalized Estimating Equations (GEE)* approach to analyze the effect of harvesting time, riboflavin concentration, UVA-light illumination and CXL on HCEC migration and proliferation in cooperation with the Institute of Medical Biometry, Epidemiology and Medical Informatics, Saarland University Medical Center, Homburg/Saar, Germany. A GEE is used to estimate the parameters of a generalized linear model with a possible

unknown correlation between outcomes. Parameter estimates from the GEE are consistent even when the covariance structure is misspecified under mild regularity conditions. The focus of the GEE is on estimating the average response over the population ("population-averaged" effects) rather than the regression parameters that would enable prediction of the effect of changing one or more covariates on a given individual.

In addition, *Mann-Whitney test* was used to

1. compare viability, migration and proliferation of HCECs using AS and FBS with the same concentration.
2. compare HCEC cultures treated by AMS or AMH.

Generalized linear model (GLM) was used to analyze

1. the effects of different concentrations of AS and the impact of growth factor concentrations in AS on HCEC viability, migration and proliferation.
2. the effects of growth factor concentrations in AMS and AMH on HCEC viability, migration and proliferation.

For this model, we calculated the concentration of the growth factors from the concentration measurement results of 100% AS, 100% AMS and AMH, but we did not perform new growth factor concentration measurements for 5, 10, 15 and 30% AS, or for 15% and 30% AMS and AMH. Even though we know that the growth factor ELISA measurement curves are in part non-linear, we still found this method a good estimate.

3 RESULTS

3.1 Effect of keratocyte supernatant on HCEC migration and proliferation after CXL

Results of HCEC migration and proliferation after CXL are displayed in **Tables 1-4** and **Figures 3-5**.

HCEC migration was 141 ± 21 µm after 9 hours using normal keratocyte supernatant harvested after 5 hours, without application of riboflavin and UVA light. Using UVA light and 0.1% riboflavin concentration for the same conditions, HCEC migration was 128 ± 14 µm.

HCEC migration was 159 ± 31 µm after 9 hours using keratoconus keratocyte supernatant harvested after 24 hours, without application of riboflavin and UVA light. Using UVA light and 0.1% riboflavin concentration for the same conditions, HCEC migration was 131 ± 26 µm.

Using a linear mixed model in the framework of a Generalized Estimating Equations (GEE), riboflavin concentration did not show significant impact on HCEC migration using normal or keratoconus keratocyte supernatant ($P > 0.10$), however, longer harvesting time of normal or keratoconus keratocyte supernatant significantly increased ($P = 0.01$ for both) and UVA-light illumination of keratoconus keratocytes ($P < 0.001$) significantly decreased HCEC migration.

Riboflavin concentration, UVA-light illumination and harvesting time of normal or keratoconus keratocyte supernatant had no significant impact on HCEC proliferation $P > 0.10$).

Table 1. HCEC migration and proliferation using normal keratocyte supernatant harvested after 5 hours. Values indicate mean ± 95% confidence intervals.

treatment	HCEC migration (μm)			HCEC proliferation (absorbance)
	3h	6h	9h	
-UVA + 0% riboflavin	66 ± 9	108 ± 14	141 ± 21	0.33 ± 0.16
-UVA + 0.05% riboflavin	58 ± 9	90 ± 18	129 ± 27	0.37 ± 0.08
-UVA + 0.10% riboflavin	61 ± 12	100 ± 12	137 ± 14	0.38 ± 0.10
+UVA + 0% riboflavin	70 ± 14	99 ± 17	132 ± 17	0.42 ± 0.12
+UVA + 0.05% riboflavin	59 ± 12	99 ± 14	133 ± 14	0.43 ± 0.15
+UVA + 0.10% riboflavin	61 ± 11	95 ± 13	128 ± 14	0.41 ± 0.11

Table 2. HCEC migration and proliferation using normal keratocyte supernatant harvested after 24 hours. Values indicate mean ± 95% confidence intervals.

treatment	HCEC migration (μm)			HCEC proliferation (absorbance)
	3h	6h	9h	
-UVA + 0% riboflavin	73 ± 21	116 ± 28	152 ± 25	0.46 ± 0.16
-UVA + 0.05% riboflavin	70 ± 9	111 ± 15	152 ± 15	0.45 ± 0.19
-UVA + 0.10% riboflavin	66 ± 9	107 ± 9	147 ± 10	0.45 ± 0.19
+UVA + 0% riboflavin	66 ± 15	109 ± 17	145 ± 21	0.41 ± 0.17
+UVA + 0.05% riboflavin	70 ± 15	112 ± 10	151 ± 12	0.45 ± 0.19
+UVA + 0.10% riboflavin	61 ± 11	0.41 ± 0.11	128 ± 14	0.41 ± 0.11

Table 3. HCEC migration and proliferation using keratoconus keratocyte supernatant harvested after 5 hours. Values indicate mean ± 95% confidence intervals.

treatment	HCEC migration (μm)			HCEC proliferation (absorbance)
	3h	6h	9h	
-UVA + 0% riboflavin	52 ± 25	100 ± 21	133 ± 20	0.34 ± 0.17
-UVA + 0.05% riboflavin	64 ± 15	97 ± 16	130 ± 16	0.26 ± 0.14
-UVA + 0.10% riboflavin	59 ± 16	108 ± 18	132 ± 15	0.27 ± 0.10
+UVA + 0% riboflavin	65 ± 12	99 ± 9	134 ± 14	0.30 ± 0.09
+UVA + 0.05% riboflavin	60 ± 15	86 ± 10	115 ± 14	0.28 ± 0.10
+UVA + 0.10% riboflavin	57 ± 14	91 ± 20	123 ± 24	0.26 ± 0.09

Table 4. HCEC migration and proliferation using keratoconus keratocyte supernatant harvested after 24 hours. Values indicate mean ± 95% confidence intervals.

treatment	HCEC migration (μm)			HCEC proliferation (absorbance)
	3h	6h	9h	
-UVA +0% riboflavin	68 ± 22	125 ± 29	159 ± 31	0.32 ± 0.07
-UVA +0.05% riboflavin	67 ± 13	116 ± 11	144 ± 9	0.25 ± 0.07
-UVA +0.10% riboflavin	77 ± 15	117 ± 16	147 ± 13	0.25 ± 0.08
+UVA +0% riboflavin	64 ± 9	99 ± 8	135 ± 11	0.24 ± 0.06
+UVA +0.05% riboflavin	54 ± 5	98 ± 16	131 ± 17	0.25 ± 0.06
+UVA +0.10% riboflavin	61 ± 11	95 ± 20	131 ± 26	0.28 ± 0.16

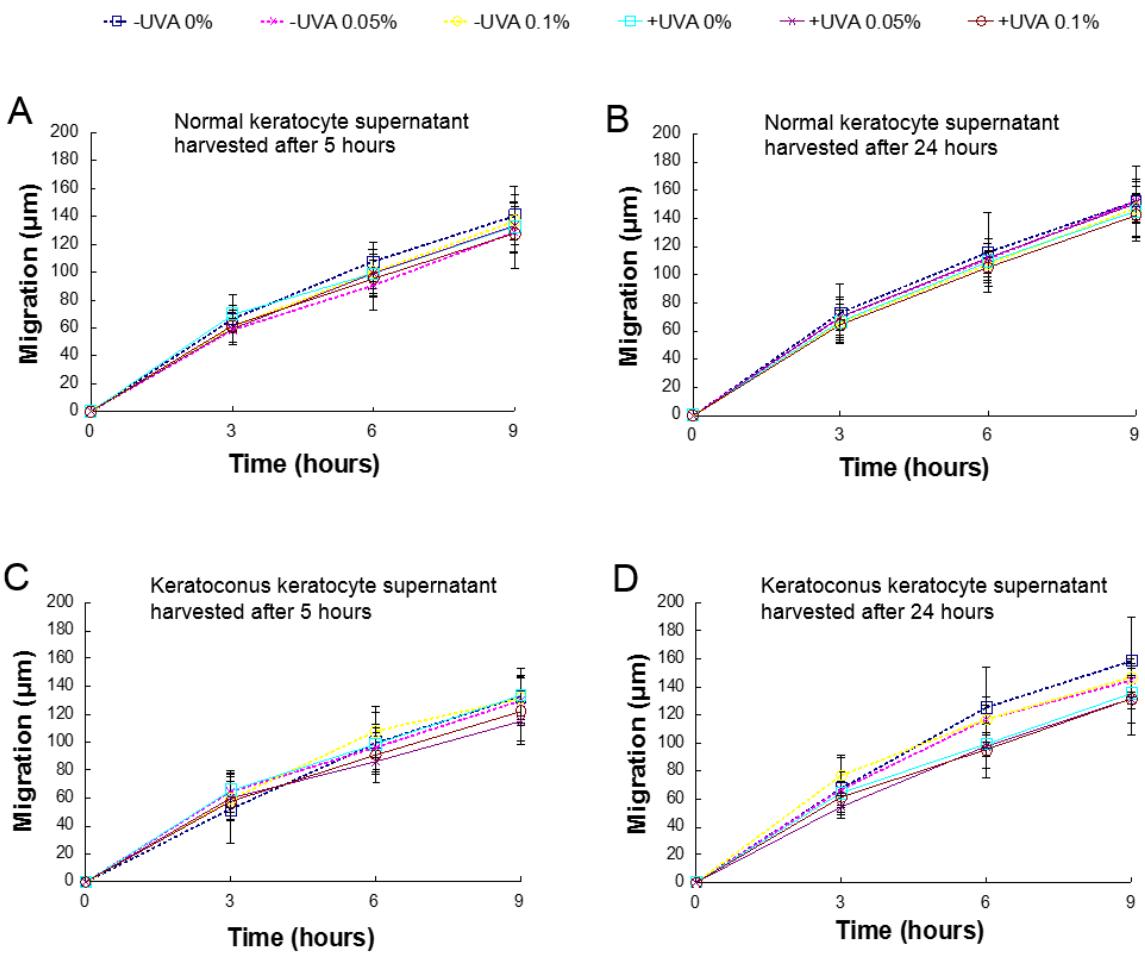

Figure 3. HCEC migration 3, 6 and 9 hours following scratch, using supernatant of normal (A, B) or keratoconus keratocytes (C, D), 5 (A, C) or 24 hours (B, D) after treatment.

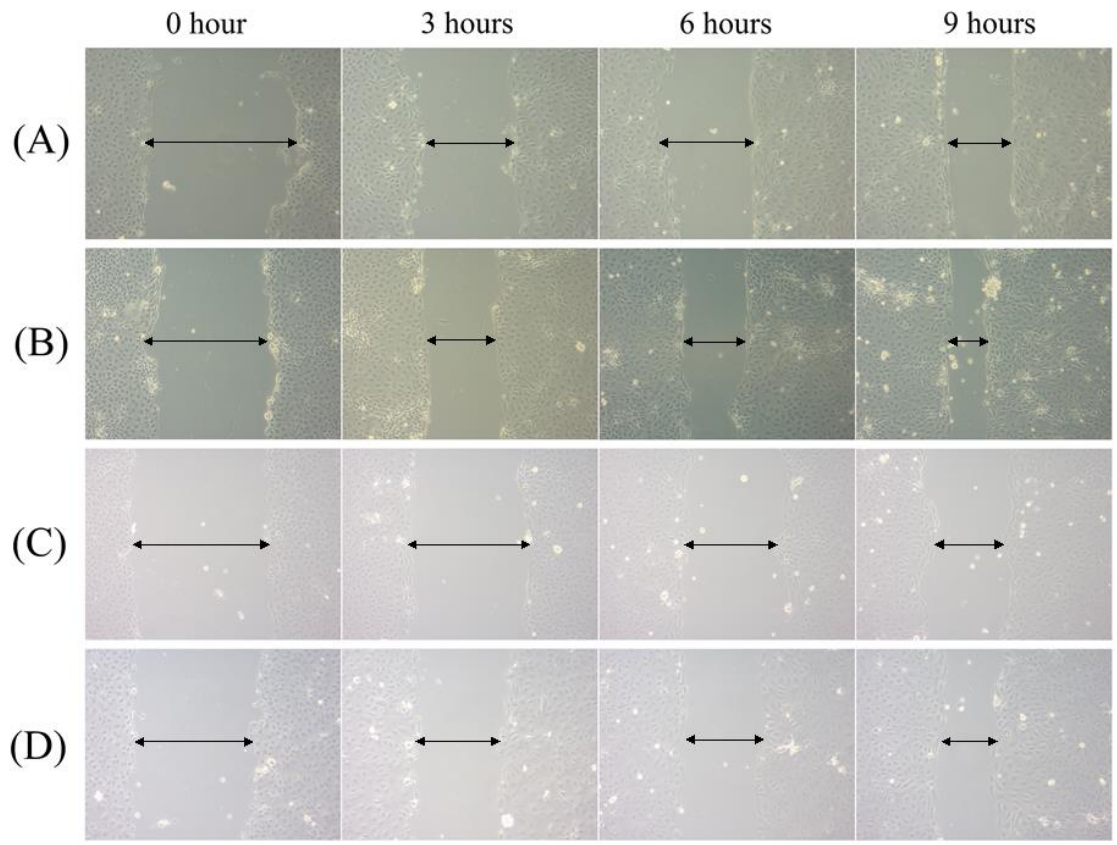

Figure 4. HCEC migration 0, 3, 6 and 9 hours following scratch, using supernatant of normal (A, B) or keratoconus keratocytes (C, D) 5 (A, C) or 24 hours (B, D) after treatment.

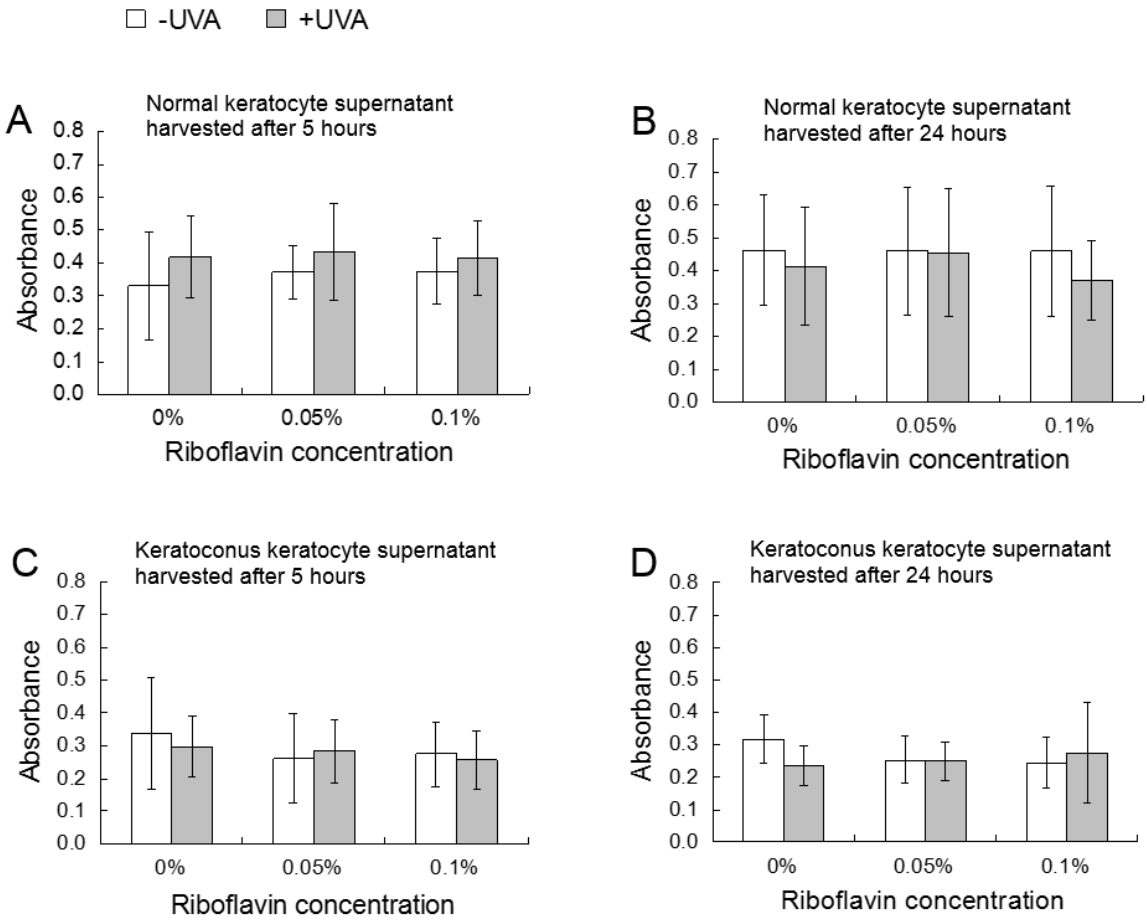

Figure 5. HCEC proliferation using supernatant of normal (A, B) or keratoconus keratocytes (C, D), 5 (A, C) or 24 hours (B, D) after treatment. Using a linear mixed model in the framework of a Generalized Estimating Equations (GEE) approach, riboflavin concentration, UVA-light illumination and harvesting time of normal or keratoconus keratocyte supernatant had no significant impact on HCEC proliferation ($P > 0.10$).

3.2 Effect of autologous serum and fetal bovine serum on human corneal epithelial cell viability, migration and proliferation

Viability, migration and proliferation of HCECs using different concentrations of AS and FBS are displayed in **Table 5** and **Figures 6-9**.

Table 5. Viability, migration and proliferation of human corneal epithelial cells (HCECs) using different concentrations of autologous serum (AS) and fetal bovine serum (FBS). Values indicate mean ± 95% confidence intervals.

	concentration	Viability (absorbance)	Migration (μm)	Proliferation (absorbance)
AS	5%	0.2423 ± 0.0318	132 ± 11	0.2609 ± 0.0290
	10%	0.1965 ± 0.0358	133 ± 15	0.2665 ± 0.0318
	15%	0.2171 ± 0.0403	136 ± 12	0.2837 ± 0.0295
	30%	0.2687 ± 0.0370	166 ± 10	0.0969 ± 0.0632
FBS	5%	0.1891 ± 0.0223	109 ± 7	0.2805 ± 0.0375
	10%	0.1893 ± 0.0155	120 ± 10	0.2484 ± 0.0426
	15%	0.2233 ± 0.0275	128 ± 10	0.2589 ± 0.0427
	30%	0.1766 ± 0.0329	144 ± 9	0.1302 ± 0.0527

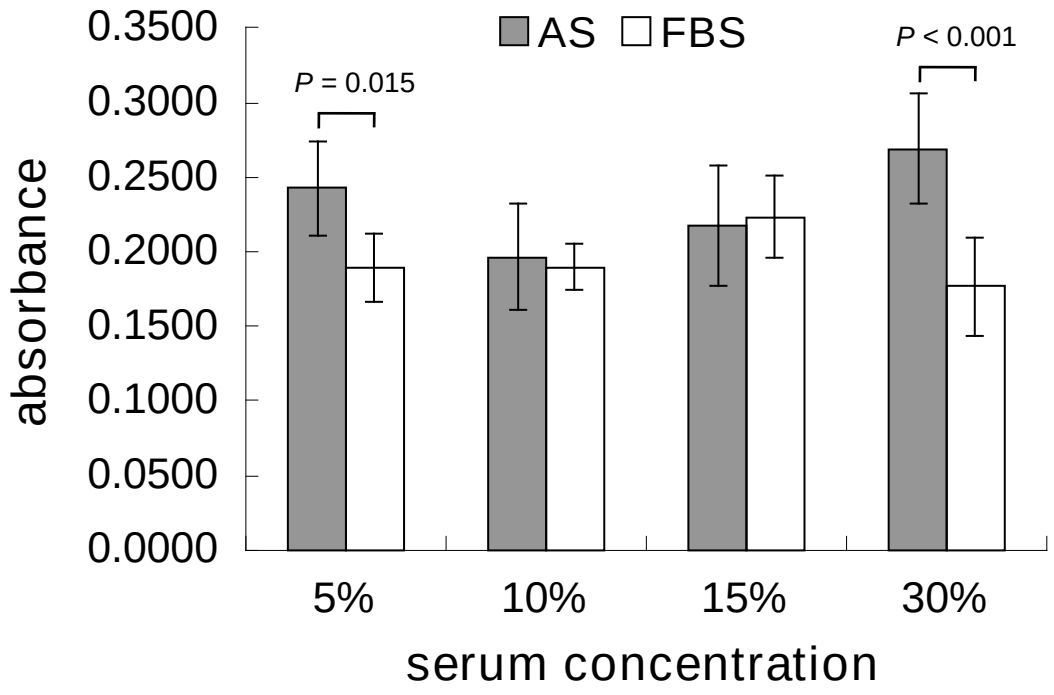

Figure 6. Human corneal epithelial cell (HCEC) viability using different concentrations of autologous serum (AS) and fetal bovine serum (FBS).

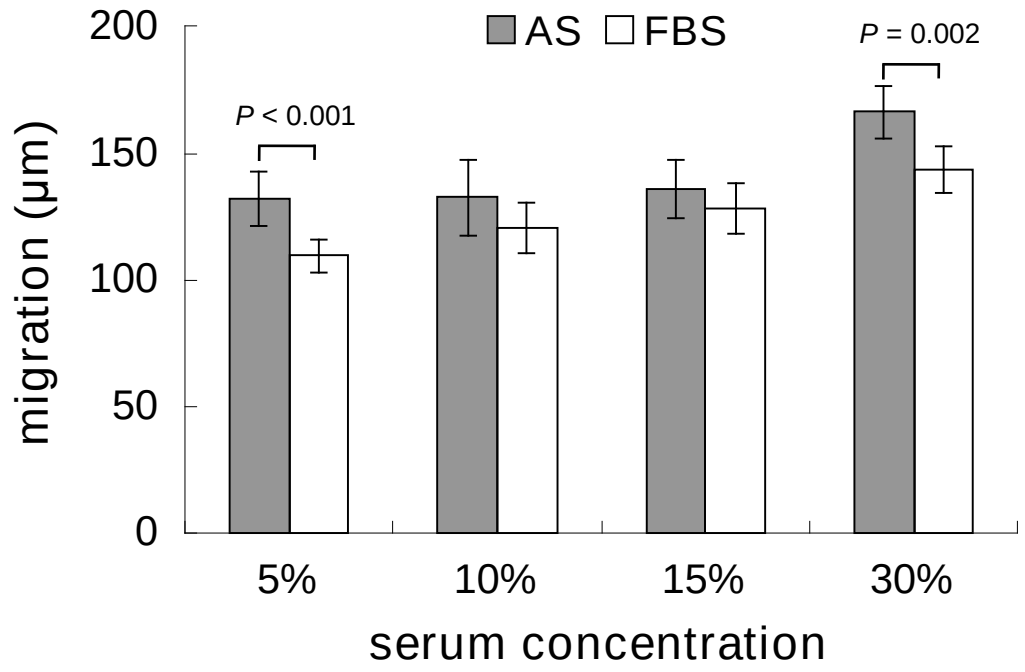

Figure 7. Human corneal epithelial cell (HCEC) migration using different concentrations of autologous serum (AS) and fetal bovine serum (FBS).

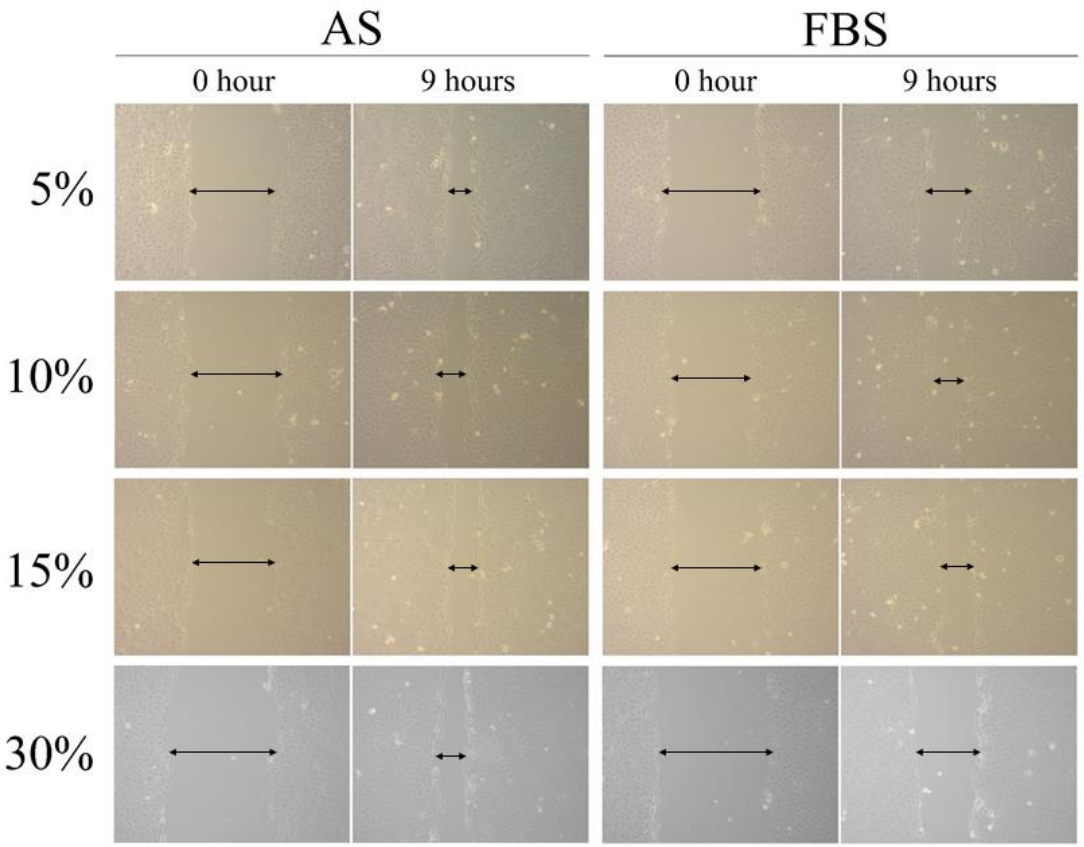

Figure 8. Human corneal epithelial cell (HCEC) migration using different concentrations of autologous serum (AS) and fetal bovine serum (FBS), 0 and 9 hours following scratch.

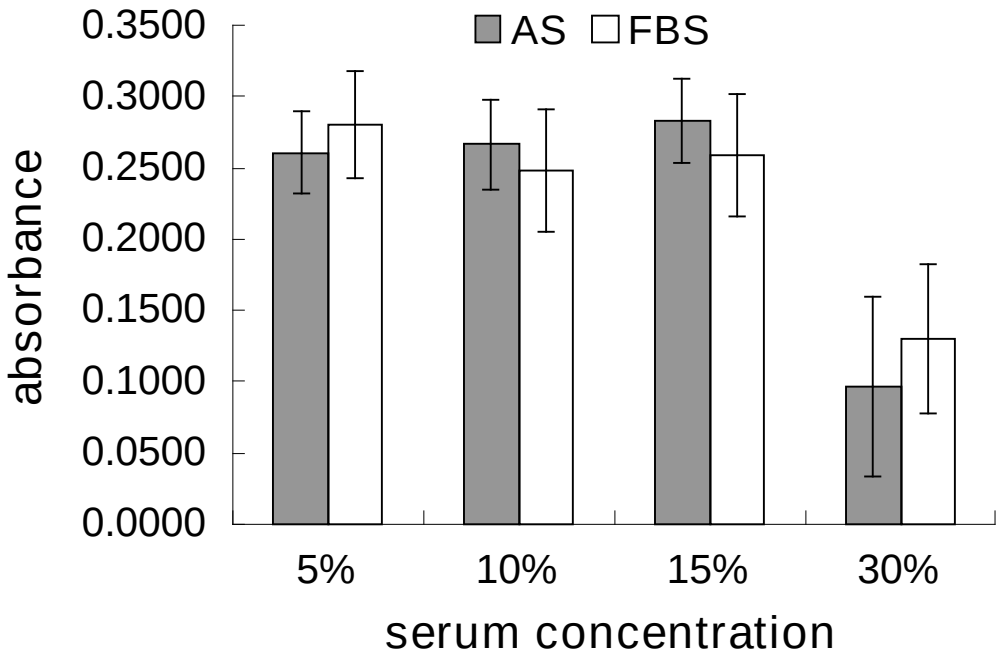

Figure 9. Human corneal epithelial cell (HCEC) proliferation using different concentrations of autologous serum (AS) and fetal bovine serum (FBS). Mann-Whitney test was used to compare AS and FB groups with the same concentration.

3.2.1 Effect of different concentrations of autologous serum and fetal bovine serum on human corneal epithelial cell viability, migration and proliferation

Effect of different concentrations of AS or FBS on HCEC viability, migration and proliferation analyzed by a generalized linear model are shown in **Table 6**.

HCEC viability was the highest at 30% AS or 15% FBS and the lowest at 10% AS or 30% FBS application. HCEC migration was the quickest through 30% AS or 30% FBS and the slowest through 5% AS or 5% FBS concentrations. Proliferation was the most increased through 15% AS or 5% FBS and the least increased through 30% AS or 30% FBS concentrations.

HCEC viability at 15% AS was significantly worse ($P = 0.023$) compared to baseline (30%) and significantly better using 15% FBS ($P = 0.003$), as 30% FBS concentrations. HCEC migration was significantly worse ($P \leq 0.007$) and HCEC proliferation significantly better ($P < 0.001$) in all concentration groups compared to baselines (30% AS and 30% FBS).

Table 6. Effect of different concentrations of autologous serum (AS) or fetal bovine serum (FBS) on human corneal epithelial cell (HCEC) viability, migration and proliferation analyzed by a generalized linear model. The values at 30% AS and 30% FBS were used as baselines (in *italic*). R.C. = regression coefficient. $P < 0.05$ was considered statistically significant, compared to baseline.

	concentration	viability		migration		proliferation	
		R.C.	*P* value	R.C.	*P* value	R.C.	*P* value
AS	5%	-0.026	0.244	-34.319	**<0.001**	0.164	**<0.001**
	10%	-0.072	**0.001**	-33.595	**<0.001**	0.170	**<0.001**
	15%	-0.052	**0.023**	-30.583	**<0.001**	0.187	**<0.001**
	30%	*0*	-	*0*	-	*0*	-
FBS	5%	0.013	0.428	-34.154	**<0.001**	0.150	**<0.001**
	10%	0.013	0.423	-23.084	**<0.001**	0.118	**<0.001**
	15%	0.047	**0.003**	-15.365	**0.007**	0.129	**<0.001**
	30%	*0*	-	*0*	-	*0*	-

3.2.2 Effect of autologous serum and fetal bovine serum with the same concentration on human corneal epithelial cell viability, migration and proliferation

Results of the Mann-Whitney test to compare viability, migration and proliferation of HCECs in AS and FBS groups with the same concentration are shown in **Table 7** and **Figures 6-9**.

Viability and migration was significantly higher using 5% AS than 5% FBS ($P = 0.015$, $P < 0.001$). Viability and migration were also significantly higher using 30% AS than 30% FBS ($P < 0.001$, $P = 0.002$). Proliferation did not differ significantly between AS and FBS groups with the same concentration ($P > 0.096$).

Table 7. Mann-Whitney test was used to compare viability, migration and proliferation of human corneal epithelial cells (HCECs) in autologous serum (AS) and fetal bovine serum (FBS) groups with the same concentration. *P* values are shown (significant values in **bold**).

	concentration	viability	migration	proliferation
AS vs FBS	5%	**0.015**	**0.001**	0.159
	10%	0.397	0.077	0.626
	15%	0.980	0.457	0.174
	30%	**0.001**	**0.002**	0.096

3.2.3 Effect of growth factors in autologous serum on viability, migration and proliferation of human corneal epithelial cells

Concentrations of KGF, FGFb, HGF and TGF-β1 in AS of 13 patients are shown in **Table 8**. The effect of FGFb, HGF and TGF-β1 concentrations in AS on HCEC viability, migration and proliferation using a generalized linear model is displayed in **Table 9**. Effect of KGF on HCEC viability, migration and proliferation was not considered in **Table 9**, since KGF was only measurable in one AS sample.

Concentration of the measured growth factors did not effect HCEC viability ($P > 0.590$). However, FGFb and HGF concentrations had a positive effect ($P < 0.001$ for both) on HCEC migration and FGFb and TGF-β1 concentrations a negative effect ($P = 0.006$, $P = 0.008$) on HCEC proliferation.

Table 8. Growth factor concentrations in autologous serum (AS) of 13 patients.

patient No.	growth factors (pg/mL)			
	KGF	FGFb	HGF	TGF-β1
1	0	202	139	2928
2	0	204	1115	2928
3	0	130	1884	2313
4	0	122	2888	3426
5	0	56	1272	3955
6	0	0	2784	16767
7	0	300	628	28906
8	106	136	5964	28218
9	0	58	1426	28873
10	0	0	1182	26046
11	0	130	308	25075
12	0	96	661	25781
13	0	131	1715	35045
median	0	130	1272	25075

Table 9. Effect of FGFb, HGF and TGF-β1 concentrations in autologous serum (AS) on human corneal epithelial cell (HCEC) viability, migration and proliferation using a generalized linear model. Effect of KGF on HCEC viability, migration or proliferation was not considered in the following table, as KGF was only measurable in one AS sample. R.C. = regression coefficient. $P < 0.05$ was considered statistically significant, compared to baseline.

growth factor	viability		migration		proliferation	
	R.C.	*P* value	R.C.	*P* value	R.C.	*P* value
FGFb	3.3×10^{-4}	0.590	0.623	**<0.001**	-0.002	**0.006**
HGF	1.74×10^{-6}	0.945	0.024	**<0.001**	-8.02×10^{-5}	0.086
TGF-β1	2.30×10^{-6}	0.666	3.14×10^{-4}	0.808	-1.50×10^{-5}	**0.008**

3.3 Effect of amniotic membrane suspension and amniotic membrane homogenate on human corneal epithelial cells

3.3.1 Effect of amniotic membrane suspension and amniotic membrane homogenate on human corneal epithelial cell viability, migration and proliferation

HCEC viability, migration and proliferation are displayed in **Table 10** and **Figures 10-13**. HCEC viability remained unchanged using 15% or 30% AMS ($P = 1.0$ for both). However, it decreased significantly using 100% AMS ($P < 0.001$) or 15% AMH ($P = 0.041$) or 30% AMH ($P < 0.001$), compared to controls.

Analyzing HCEC migration, using 30% AMH containing media, two membrane groups had to be excluded from further cell migration analysis, causing severe HCEC detachment. Data using only the remaining groups are described below.

Using 15% or 30% AMS, HCEC migration increased significantly ($P < 0.001$ for both), compared to controls. Using 15% or 30% AMH ($P = 0.153$; P = 0.083), HCEC migration remained unchanged and 100% AMS inhibited HCEC migration ($P < 0.001$). 15% and 30% AMS had no effect on HCEC proliferation ($P = 0.454$ and $P = 0.119$), but 100% AMS ($P < 0.001$) and 15% ($P = 0.002$) and 30% AMH ($P = 0.001$) inhibited HCEC proliferation significantly, compared to controls.

There was no significant difference in HCEC viability, migration and proliferation comparing 15% and 30% AMS or 15% and 30% AMH ($P > 0.248$).

Table 10. Human corneal epithelial cell (HCEC) viability, migration and proliferation using amniotic membrane suspension (AMS) or amniotic membrane homogenate (AMH) containing media (values indicate mean ± SD). A culture medium containing DMEM/F12 + 5% FBS + 1% P/S was used as control. Analyzing HCEC migration, using 30% AMH containing media (*italics*), two membrane groups had to be excluded from further cell migration analysis, causing severe HCEC detachment. Data using only the remaining groups are described below.

	HCEC viability (absorbance)	HCEC migration (μm)	HCEC proliferation (absorbance)
control	0.2988 ± 0.0902	121 ± 20	0.4186 ± 0.1029
15% AMS	0.2961 ± 0.0562	171 ± 18	0.3372 ± 0.1266
30% AMS	0.2951 ± 0.0534	173 ± 28	0.3468 ± 0.0970
100% AMS	0.0976 ± 0.0448	62 ± 21	0.0514 ± 0.0524
15% AMH	0.1966 ± 0.1110	133 ± 28	0.2443 ± 0.1419
30% AMH	0.1382 ± 0.0927	*141 ± 31*	0.1854 ± 0.1699

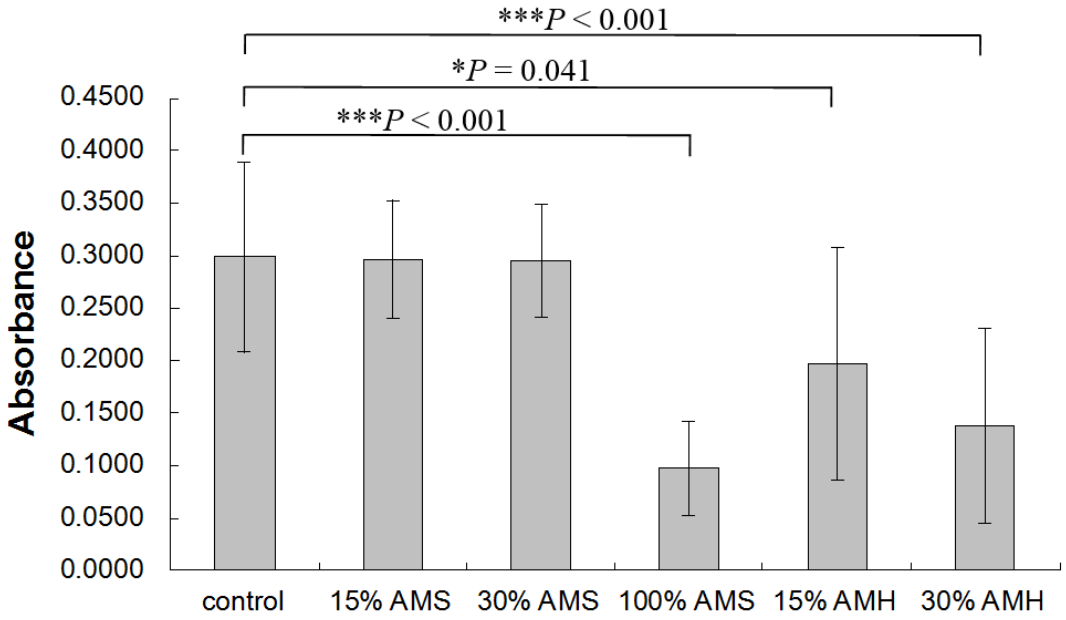

Figure 10. Human corneal epithelial cell (HCEC) viability using different concentrations of amniotic membrane suspension (AMS) and amniotic membrane homogenate (AMH). Significant differences compared to the control group are indicated.
A culture medium containing DMEM/F12 + 5% FBS + 1% P/S was used as control.

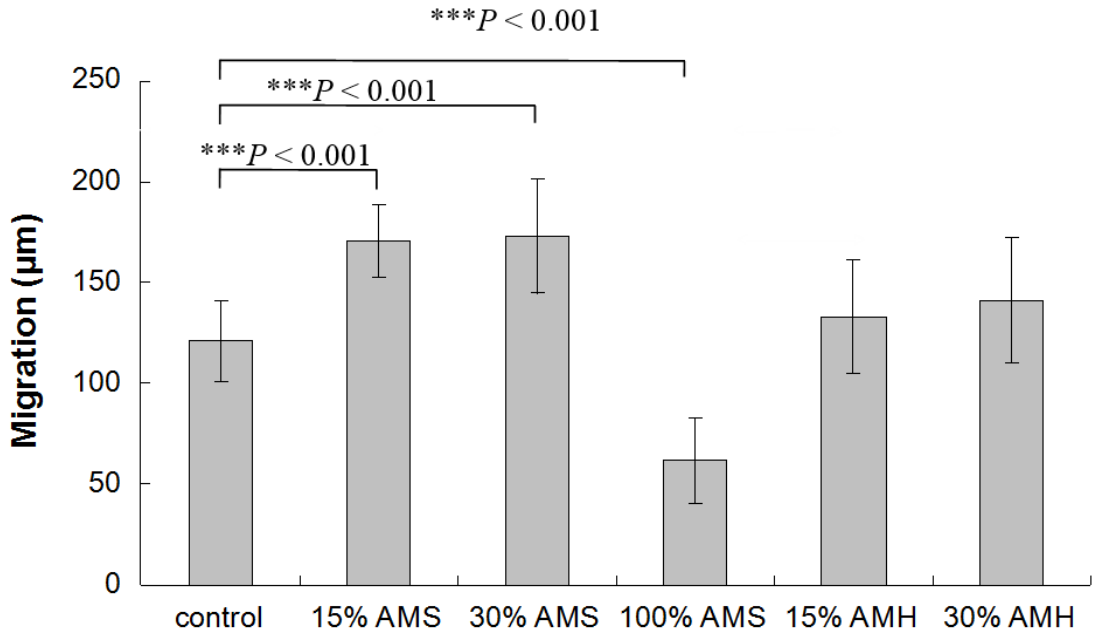

Figure 11. Human corneal epithelial cell (HCEC) migration using different concentrations of amniotic membrane suspension (AMS) and amniotic membrane homogenate (AMH). Significant differences compared to the control group are indicated. A culture medium containing DMEM/F12 + 5% FBS + 1% P/S was used as control. Analyzing HCEC migration, using 30% AMH containing media, two membranes, causing severe HCEC detachment had to be excluded from the analysis.

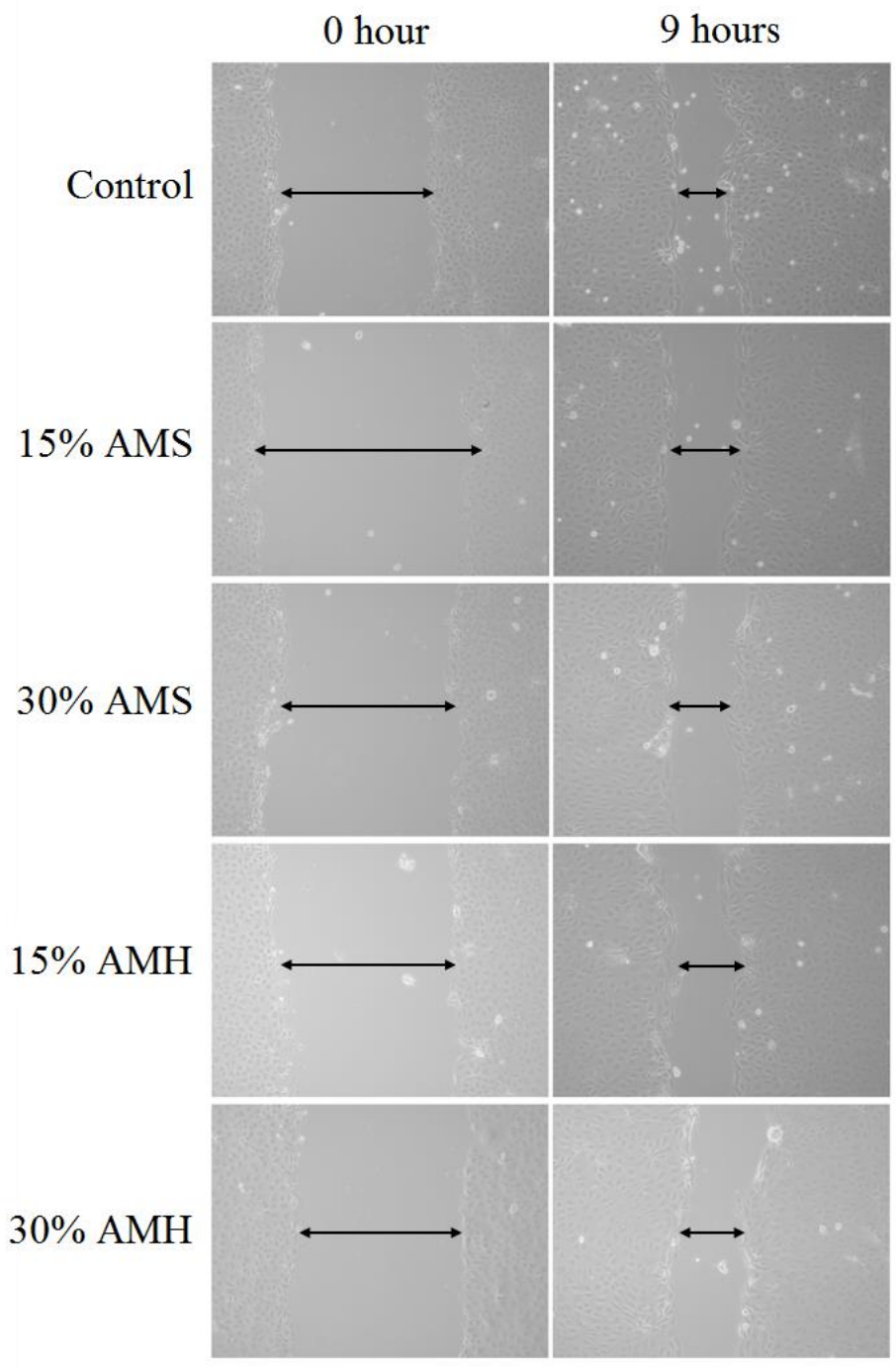

Figure 12. Human corneal epithelial cell (HCEC) migration using different concentrations of amniotic membrane suspension (AMS) and amniotic membrane homogenate (AMH) 0 and 9 hours after scratch.

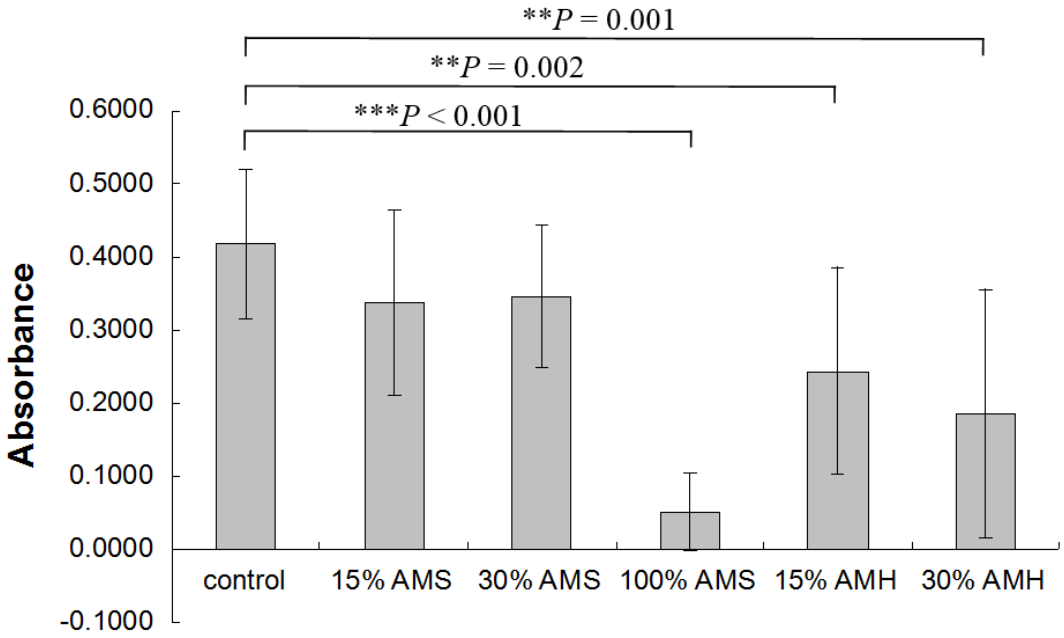

Figure 13. Human corneal epithelial cell (HCEC) migration using different concentrations of amniotic membrane suspension (AMS) and amniotic membrane homogenate (AMH). Significant differences compared to the control group are indicated. A culture medium containing DMEM/F12 + 5% FBS + 1% P/S was used as control.

3.3.2 Impact of growth factor concentrations in amniotic membrane suspension and amniotic membrane homogenate on human corneal epithelial cell viability, migration and proliferation

KGF, FGFb, HGF and TGF-β1 concentrations in 100% AMS (n = 2) or AMH (n = 6) are shown in **Table 11**. Impact of growth factor concentrations in AMS and AMH on HCEC viability, migration and proliferation are shown in **Table 12**.

FGFb concentration had a significant negative effect on HCEC migration ($P = 0.002$), but also a significant positive effect on HCEC viability ($P = 0.02$) and TGF-β1 concentration had a significant positive effect on HCEC proliferation ($P < 0.001$). Using a generalized linear model, no other significant effects of growth factor concentrations could be shown on HCEC viability, migration and proliferation ($P > 0.08$).

Table 11. Growth factor concentrations in 100% amniotic membrane suspension (AMS) in 2 of the used amnion donors and in 100% amniotic membrane homogenate (AMH) in 6 of the used donors.

placenta	growth factors (pg/mL)			
	KGF	FGFb	HGF	TGF-β1
1 (AMS)	71	299	12162	257
2 (AMS)	62	251	11109	562
3 (AMH)	34	320	2822	334
4 (AMH)	240	466	8634	336
5 (AMH)	18	361	3405	335
6 (AMH)	185	1913	10276	468
7 (AMH)	0	172	2848	257
8 (AMH)	27	136	7070	270

Table 12. Impact of FGFb, HGF, TGF-β1 concentrations in amniotic membrane suspension (AMS) and amniotic membrane homogenate (AMH) on human corneal epithelial cell (HCEC) viability, migration and proliferation (generalized linear model).

R.C. = regression coefficient, $P < 0.05$ is considered statistically significant and labeled in **bold**.

growth factor	viability		migration		proliferation	
	R.C.	*P* value	R.C.	*P* value	R.C.	*P* value
KGF	0.001	0.757	0.721	0.114	-0.003	0.221
FGFb	-4.78×10^{-4}	**0.020**	-0.163	**0.002**	-2.01×10^{-4}	0.456
HGF	7.77×10^{-5}	0.141	0.009	0.522	1.20×10^{-4}	0.084
TGF-β1	0.001	0.275	-0.191	0.397	0.005	**<0.001**

4 Discussion

4.1 Effect of keratocyte supernatant on human corneal epithelial cell migration and proliferation after crosslinking

Although CXL is in clinical use since several years, to the best of our knowledge, the impact of CXL on epithelial wound healing, has not been described, yet. It is only known that CXL may inhibit the regeneration of human limbal epithelial cells [63].

The interactions between keratocytes and CECs are decisive in wound healing and corneal homeostasis. Growth factors such as the HGF and KGF secreted by the keratocytes regulate functions of HCECs. Cytokines such as interleukin-1 (IL-1), produced by HCECs, influence keratocyte functions [8]. In our experimental setup, we tried to model the impact of keratocytes on the remaining CECs, following CXL treatment, in the short-term.

The most conspicuous finding of our experimental study is that riboflavin concentration, UVA-light illumination of CXL and harvesting time of normal or keratoconus keratocyte supernatant have no significant effect on HCEC proliferation.

Interestingly, riboflavin concentration has also no significant effect on HCEC migration neither using normal nor using keratoconus keratocyte supernatant.

However, longer harvesting time of normal or keratoconus keratocyte supernatant significantly increased and UVA-light illumination of keratoconus keratocyte supernatant significantly decreased HCEC migration.

In previous experimental studies we performed CXL of keratoconus keratocytes. Interestingly, CXL decreased viability, triggered apoptosis and inhibited proliferation, however, did not have an impact on multipotent haematopoietic stem cell transformation and myofibroblastic transformation of human keratoconus keratocytes [106]. CXL also triggered FGFb secretion of keratoconus keratocytes transiently (five hours), which normalized after 24 hours. However, CXL did not have an impact on HGF, TGFβ1, VEGF, KGF, IL-1β, IL-6, and IL-8 secretion of keratoconus keratocytes in the short term [106].

The results of these previous studies show, that normal or keratoconus keratocyte secretion of different growth factors changes in the short term (from 5 to 24 hours after

CXL), which may be related to our present findings that longer harvesting time of keratocyte supernatant after treatment increases significantly HCEC migration.
This is also in accordance with the clinical observation that reepithelization may be promoted through CXL even in nonhealing epithelial defects [42, 43]. However, other still undefined factors have to be defined with effect on epithelial healing.
Our present results, show, that UVA-light illumination of keratoconus keratocytes may significantly inhibit HCEC migration. It is known that UVA-light illumination itself may cause DNA damage of the cells [107]. Interestingly, some other authors reported on occurrence of early microbial keratitis after CXL therapy [108-110]. The significantly decreased epithelial cell migration due to the effect of UVA-light illumination, using keratoconus keratocytes, may be related to this clinical observation. Kim et al. and Kaldawy et al. [111] described that after CXL an increased percentage of apoptotic keratocytes is present in human keratoconus corneas compared to normal human controls without treatment. Macé et al. [112] suggested that cell loss resulting from antiproliferative and hyperapoptotic phenotypes may be responsible for the pathogenesis of keratoconus. In addition, changes in corneal protein pattern, increase in enzymatic activities and cell apoptosis are also thought to be part of keratoconus progression [113-115]. Chwa et al. [116] described an increased basal generation of reactive oxygen species and reactive nitrogen species in keratoconus keratocytes. In addition, it is also described that cyclobutane pyrimidine dimers are induced through ultraviolet light illumination of the cornea, which may lead to DNA damage [117].
In summary harvesting time, riboflavin concentration and UV-A-light illumination of normal and keratoconus keratocyte cultures have no impact on proliferation of HCECs, in the short term. However, 24 hours harvesting time (both for normal and keratoconus keratocytes) increases and UVA-light illumination of keratoconus keratocyte cultures decreases HCEC migration.

4.2 Effect of autologous serum and fetal bovine serum on human corneal epithelial cell viability, migration and proliferation

AS has been used as a substitute for FBS in cultures of various cells, and supported better cell confluence, enhanced differentiation of bone marrow mesenchymal cells and increased cell proliferation rate, more compared to FBS [118-123]. However, only a

few studies tried to compare differences between AS and FBS in HCEC culture, and until now, no significant differences between both have been determined.

In our present study, HCEC viability and migration was better using AS than FBS. However, concerning proliferation, no difference could be shown between both groups. HCEC viability and migration were the highest at 30% AS, but this group increased HCEC proliferation the least. 15% AS concentration led to lower HCEC viability and migration than 30% AS, but 15% AS resulted in the best proliferation of the HCECs.

Based on our results, we suggest the clinical use of 30% AS, since the most important corneal epithelial functions in vivo are migration and viability. This concentration could be reached through dilution of AS in the remaining tear film of the patients. Therefore, application of AS eye drops should also be planned depending on individual tear film volume. Nevertheless, the high variability of growth factor concentrations in AS of different patients, should also be taken into account.

It is already known that FGFb is a beneficial factor in CEC growth or corneal wound healing [23, 124], and it is also required for CEC proliferation and differentiation during embryonic development [125]. HGF and FGFb are both major factors initiating proliferation and migration in the cornea, while TGF-β in the tear film suppresses the proliferation at the migrating cell front [126].

In the present study we analyzed the effects of FGFb, HGF and TGF-β1 concentrations on HCEC viability, migration and proliferation. FGFb and HGF concentrations had a positive effect on HCEC migration, but FGFb and TGF-β1 concentrations had a negative effect on HCEC proliferation. In our opinion, HCEC proliferation might be inhibited through high concentrations of FGFb in 30% AS.

In summary, HCEC viability is mostly increased through 30% AS or 15% FBS, migration through 30% AS or 30% FBS and proliferation through 15% AS or 5% FBS. In addition, AS better supports HCECs viability and migration than FBS. Therefore, we suggest the use of 30% AS in the clinical practice. Based on our experiments, we also suggest the use of AS instead of FBS for in vitro HCEC cultures, especially for ex vivo expansion of limbal stem cells.

4.3 Effect of amniotic membrane suspension and amniotic membrane homogenate on human corneal epithelial cell viability, migration and proliferation

Amniotic membrane is accepted as a beneficial tissue in ocular surgery since scientists and physicians understood that it could promote epithelialization, inhibit fibrosis, reduce inflammation and angiogenesis, have antimicrobial and antiviral properties [95]. Amniotic membrane does not only provide a mechanical substrate for HCECs to migrate on, but also effect morphology [127, 128], differentiation, stratification [129, 130], stemness [131, 132] and gene expression [133-135] of HCECs.

Long-term follow-up of amniotic membrane transplantation shows that the ocular use of amniotic membrane is safe [136-138]. Clinical use of an amniotic membrane "extract" was also shown to be safe in previous studies and no side effects could be detected [96, 97, 139].

As a positive effect, in our present study we found that both 15% and 30% AMS promote HCEC migration without changes in HCEC viability and proliferation. We suggest the future use of these concentrations in clinical practice, which could be reached through dilution of AMS in the tear film.

Amniotic epithelial cells produce growth factors, such as EGF, TGF-α, KGF, HGF, FGFb, TGF-β1 and TGF-β2 [87], and neurotrophic factors, neurotransmitters and a so-called pigment epithelium-derived factor (PEDF) [140-142]. These factors may also be detected following cryopreservation of the amniotic membrane, even though the amniotic epithelial cells are not present any more [143]. It is already known that HGF, KGF and FGF are the major factors initiating proliferation and migration of CECs, while TGF-β in the tear film suppresses the proliferation at the migrating cell front [126]. In the present study, we measured a HGF concentration above 2822 pg/mL, and a KGF under 240 pg/mL, FGF under 1913 pg/mL and TGF-β1 under 468 pg/mL in 100% AMH using ELISA. We also found that FGFb may play a negative role, while TGF-β1 may play a positive role in regulating HCEC functions.

Nevertheless, we have to remark, that there must be several factors, not analyzed in this study or even not yet known, with an impact on HCEC viability, migration and proliferation. These factors should be further recognized and analyzed.

As a negative effect, 15% and 30% AMH reduces HCEC viability and inhibit

proliferation, while 100% AMS reduces HCEC viability and inhibits both migration and proliferation.

Interestingly, some previous studies, analyzing cells not derived from the cornea, have also found general inhibiting effect of amniotic membrane on various cells. Amniotic membrane could inhibit functions of conjunctival epithelial cells [144], vascular endothelial cells [142] and leukocytes [145, 146]. Furthermore, amniotic membrane could even suppress the growth of cancer cells [147, 148]. Mamede et al. used the MTT assay to evaluate the effects of human amniotic membrane protein extracts on 21 cancer cell lines and in 14 of these, the metabolic activities were inhibited [149]. Magatti et al. found that amniotic mesenchymal tissue cells reduced the proliferation of cancer cell lines of haematopoietic and non-haematopoietic origin, in co-cultures [150].

These studies suggest that biochemical factors released from amniotic membranes may have complicated, possibly non-linear, but dose-dependent effects on HCECs. Higher concentration of amnion-derived biochemical factors, present at 100% AMS and after homogenization in AMH, could even result in inhibition of HCEC migration and proliferation. Nevertheless, dilution of the amnion-derived biochemical factors in 15 and 30% AMS supports HCEC migration. The use of lower concentration of AMS or AMH, may reduce the potential inhibiting effect of amniotic biochemical factors on HCECs. However, further research is needed focusing on concentration of growth factors or other compounds in the amniotic membrane which may inhibit HCEC functions, in order to optimize “non-surgical applications” of the amniotic membrane.

In summary, 100% AMS decreases HCEC viability, migration and proliferation. Fifteen% and 30% AMH decreases HCEC viability and proliferation and has no impact on HCEC migration. In AMS/AMH, FGFb concentration has a negative effect on HCEC migration, a positive effect on HCEC viability and TGF-β1 concentration has a positive effect on HCEC proliferation.

4.4 Conclusions and outlook to the future

In our study we determined that harvesting time, riboflavin concentration and UVA-light illumination of normal and keratoconus keratocyte cultures has no impact on proliferation of HCECs, in the short term. However, 24 hours harvesting time (both for normal and keratoconus keratocytes) increases and UVA-light-illumination of keratoconus keratocyte cultures decreases HCEC migration. We suggest that the keratoconus keratocytes may be more sensible for DNA damage. In addition, UVA illumination may induce cyclobutane pyrimidine dimers in the keratocyte supernatant added to the HCECs, and inhibit corneal epithelial migration. This should be analyzed in further experiments including other factors which may contribute to epithelial migration following UVA-light illumination.
Our experiments show, that through CXL, epithelial wound healing could be supported through increased proliferation of CECs, at least in normal corneas. Therefore, CXL could be a potential treatment option for patients with non-healing epithelial defects. To prove the effect of CXL on epithelial healing, animal experiments and clinical studies should be performed in the future including different clinical disease entities.

HCEC viability is mostly increased through 30% AS or 15% FBS, migration through 30% AS or 30% FBS and proliferation through 15% AS or 5% FBS. In addition, AS better supports HCECs viability and migration than FBS. Therefore, 30% AS could be the best for clinical practice. Based on our experiments, we also suggest the use of AS instead of FBS for in vitro HCEC cultures, especially for ex vivo expansion of limbal stem cells. Which factors in AS support epithelial wound healing beside different growth factors, should be further analyzed. Animal experiments and clinical studies should further strengthen results of our in vitro study (at least with different concentrations of serum) in the future.

With unchanged HCEC viability and proliferation and increased HCEC migration, 15% and 30% AMS application seems to be the most appropriate method to support epithelial healing. Which factors are decisive in AMS and AMH beside different growth factors for epithelial wound healing should be further analyzed.

We have demonstrated that these biomaterials are promising for clinical use, and

pointed out their potential side effects. However, like in all in vitro studies, differences between the experimental setup and the human body environment are inevitable and should not be ignored. Further in vivo animal or human experiments should verify our findings.

5 REFERENCES

1. Lehrer MS, Sun TT, Lavker RM: Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. J Cell Sci 1998; 111:2867-2875.
2. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM: Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate - implications on epithelial stem-cells. Cell 1989; 57:201-209.
3. Li W, Hayashida Y, Chen YT, Tseng SCG: Niche regulation of corneal epithelial stem cells at the limbus. Cell Res 2007; 17:26-36.
4. Castro-Munozledo F, Gomez-Flores E: Challenges to the study of asymmetric cell division in corneal and limbal epithelia. Exp Eye Res 2011; 92:4-9.
5. Yoon JJ, Ismail S, Sherwin T: Limbal stem cells: Central concepts of corneal epithelial homeostasis. World J Stem Cells 2014; 6:391-403.
6. Brewitt H: Sliding of epithelium in experimental corneal wounds. A scanning electron microscopic study. Acta Ophthalmol (Copenh) 1979; 57:945-958.
7. Crosson CE, Klyce SD, Beuerman RW: Epithelial wound closure in the rabbit cornea. A biphasic process. Invest Ophthalmol Vis Sci 1986; 27:464-473.
8. Wilson SE, Liu JJ, Mohan RR: Stromal-epithelial interactions in the cornea. Prog Retin Eye Res 1999; 18:293-309.
9. Sotozono C, Kinoshita S, Kita M, Imanishi J: Paracrine role of keratinocyte growth factor in rabbit corneal epithelial cell growth. Exp Eye Res 1994; 59:385-391.
10. Wilson SE, Walker JW, Chwang EL, He YG: Hepatocyte growth-factor, keratinocyte growth-factor, their receptors, fibroblast growth-factor receptor-2, and the cells of the cornea. Invest Ophthalmol Vis Sci 1993; 34:2544-2561.
11. Wilson SE, Chen L, Mohan RR, Liang QW, Liu J: Expression of HGF, KGF, EGF and receptor messenger RNAs following corneal epithelial wounding. Exp Eye Res 1999; 68:377-397.
12. Sotozono C, Inatomi T, Nakamura M, Kinoshita S: Keratinocyte growth factor accelerates corneal epithelial wound healing in vivo. Invest Ophthalmol Vis Sci 1995; 36:1524-1529.
13. Wilson SE, He YG, Weng J, Zieske JD, Jester JV, Schultz GS: Effect of epidermal

growth factor, hepatocyte growth factor, and keratinocyte growth factor, on proliferation, motility and differentiation of human corneal epithelial cells. Exp Eye Res 1994; 59:665-678.

14. Li Q, Weng J, Mohan RR, Bennett GL, Schwall R, Wang ZF, Tabor K, Kim J, Hargrave S, Cuevas KH, Wilson SE: Hepatocyte growth factor and hepatocyte growth factor receptor in the lacrimal gland, tears, and cornea. Invest Ophthalmol Vis Sci 1996; 37:727-739.
15. Daniels JT, Limb GA, Saarialho-Kere U, Murphy G, Khaw PT: Human corneal epithelial cells require MMP-1 for HGF-mediated migration on collagen I. Invest Ophthalmol Vis Sci 2003; 44:1048-1055.
16. McBain VA, Forrester JV, McCaig CD: HGF, MAPK, and a small physiological electric field interact during corneal epithelial cell migration. Invest Ophthalmol Vis Sci 2003; 44:540-547.
17. Yanai R, Yamada N, Inui M, Nishida T: Correlation of proliferative and anti-apoptotic effects of HGF, insulin, IGF-1, IGF-2, and EGF in SV40-transformed human corneal epithelial cells. Exp Eye Res 2006; 83:76-83.
18. Kakazu A, Chandrasekher G, Bazan HE: HGF protects corneal epithelial cells from apoptosis by the PI-3K/Akt-1/Bad- but not the ERK1/2-mediated signaling pathway. Invest Ophthalmol Vis Sci 2004; 45:3485-3492.
19. Carrington LM, Boulton M: Hepatocyte growth factor and keratinocyte growth factor regulation of epithelial and stromal corneal wound healing. J Cataract Refract Surg 2005; 31:412-423.
20. Gan L, Fagerholm P, Palmblad J: Expression of basic fibroblast growth factor in rabbit corneal alkali wounds in the presence and absence of granulocytes. Acta Ophthalmol Scand 2005; 83:374-378.
21. Pancholi S, Tullo A, Khaliq A, Foreman D, Boulton M: The effects of growth factors and conditioned media on the proliferation of human corneal epithelial cells and keratocytes. Graefes Arch Clin Exp Ophthalmol 1998; 236:1-8.
22. Assouline M, Hutchinson C, Morton K, Mascarelli F, Jeanny JC, Fayein N, Pouliquen Y, Courtois Y: In vivo binding of topically applied human bFGF on rabbit corneal epithelial wound. Growth Factors 1989; 1:251-261.
23. Hu C, Ding Y, Chen J, Liu D, Zhang Y, Ding M, Wang G: Basic fibroblast growth factor stimulates epithelial cell growth and epithelial wound healing in canine corneas. Vet Ophthalmol 2009; 12:170-175.

24. Nishida K, Kinoshita S, Yokoi N, Kaneda M, Hashimoto K, Yamamoto S: Immunohistochemical localization of transforming growth factor-beta 1, -beta 2, and -beta 3 latency-associated peptide in human cornea. Invest Ophthalmol Vis Sci 1994; 35:3289-3294.
25. Saika S: TGFbeta pathobiology in the eye. Lab Invest 2006; 86(2):106-115.
26. Haber M, Cao Z, Panjwani N, Bedenice D, Li WW, Provost PJ: Effects of growth factors (EGF, PDGF-BB and TGF-beta 1) on cultured equine epithelial cells and keratocytes: implications for wound healing. Vet Ophthalmol 2003, 6:211-217.
27. Honma Y, Nishida K, Sotozono C, Kinoshita S: Effect of transforming growth factor-beta1 and -beta 2 on in vitro rabbit corneal epithelial cell proliferation promoted by epidermal growth factor, keratinocyte growth factor, or hepatocyte growth factor. Exp Eye Res 1997; 65:391-396.
28. Andresen JL, Ledet T, Ehlers N: Keratocyte migration and peptide growth factors: the effect of PDGF, bFGF, EGF, IGF-I, aFGF and TGF-beta on human keratocyte migration in a collagen gel. Curr Eye Res 1997; 16:605-613.
29. Carrington LM, Albon J, Anderson I, Kamma C, Boulton M: Differential regulation of key stages in early corneal wound healing by TGF-beta Isoforms and their inhibitors. Invest Ophth Vis Sci 2006; 47:1886-1894.
30. Saika S, Yamanaka O, Okada Y, Tanaka S, Miyamoto T, Sumioka T, Kitano A, Shirai K, Ikeda K: TGF beta in fibroproliferative diseases in the eye. Front Biosci (Schol Ed) 2009; 1:376-390.
31. O'Kane S, Ferguson MW: Transforming growth factor beta s and wound healing. Int J Biochem Cell Biol 1997; 29:63-78.
32. Chen S, Mienaltowski MJ, Birk DE: Regulation of corneal stroma extracellular matrix assembly. Exp Eye Res 2015; 133:69-80.
33. Kagan HM, Trackman PC: Properties and function of lysyl oxidase. Am J Respir Cell Mol Biol 1991; 5:206-210.
34. Spoerl E, Seiler T: Techniques for stiffening the cornea. J Refract Surg 1999; 15:711-713.
35. Raiskup F, Spoerl E: Corneal crosslinking with riboflavin and ultraviolet A. I. Principles. Ocul Surf 2013; 11:65-74.
36. Wollensak G, Spoerl E, Seiler T: Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. Am J Ophthalmol 2003; 135:620-627.

37. Ziaei M, Barsam A, Shamie N, Vroman D, Kim T, Donnenfeld ED, Holland EJ, Kanellopoulos J, Mah FS, Randleman JB, Daya S, Güell J, ASCRS Cornea Clinical Committee: Reshaping procedures for the surgical management of corneal ectasia. J Cataract Refract Surg 2015; 41:842-872.
38. Greenstein SA, Shah VP, Fry KL, Hersh PS: Corneal thickness changes after corneal collagen crosslinking for keratoconus and corneal ectasia: one-year results. J Cataract Refract Surg 2011; 37:691-700.
39. Makdoumi K, Mortensen J, Sorkhabi O, Malmvall BE, Crafoord S: UVA-riboflavin photochemical therapy of bacterial keratitis: a pilot study. Graefes Arch Clin Exp Ophthalmol 2012; 250:95-102.
40. Price MO, Tenkman LR, Schrier A, Fairchild KM, Trokel SL, Price FW: Photoactivated riboflavin treatment of infectious keratitis using collagen cross-linking technology. Journal of Refractive Surgery 2012; 28:706-713.
41. Khan YA, Kashiwabuchi RT, Martins SA, Castro-Combs JM, Kalyani S, Stanley P, Flikier D, Behrens A: Riboflavin and ultraviolet light A therapy as an adjuvant treatment for medically refractive acanthamoeba keratitis report of 3 cases. Ophthalmology 2011; 118:324-331.
42. Iseli HP, Thiel MA, Hafezi F, Kampmeier J, Seiler T: Ultraviolet A/riboflavin corneal cross-linking for infectious keratitis associated with corneal melts. Cornea 2008; 27:590-594.
43. Makdoumi K, Mortensen J, Crafoord S: Infectious keratitis treated with corneal crosslinking. Cornea 2010; 29(12):1353-1358.
44. Brem R, Karran P: Multiple forms of DNA damage caused by UVA photoactivation of DNA 6-thioguanine. Photochem Photobiol 2012; 88:5-13.
45. Cardoso DR, Libardi SH, Skibsted LH: Riboflavin as a photosensitizer. Effects on human health and food quality. Food Funct 2012; 3:487-502.
46. Jordan C, Patel DV, Abeysekera N, McGhee CNJ: In vivo confocal microscopy analyses of corneal microstructural changes in a prospective study of collagen cross-linking in keratoconus. Ophthalmology 2014; 121:469-474.
47. Messmer EM, Meyer P, Herwig MC, Loeffler KU, Schirra F, Seitz B, Thiel M, Reinhard T, Kampik A, Auw-Haedrich C: Morphological and immunohistochemical changes after corneal cross-linking. Cornea 2013; 32:111-117.
48. Mencucci R, Marini M, Paladini I, Sarchielli E, Sgambati E, Menchini U,

Vannelli GB: Effects of riboflavin/UVA corneal cross-linking on keratocytes and collagen fibres in human cornea. Clin Experiment Ophthalmol 2010; 38:49-56.

49. Caporossi A, Mazzotta C, Baiocchi S, Caporossi T, Paradiso AL: Transepithelial corneal collagen crosslinking for keratoconus: qualitative investigation by in vivo HRT II confocal analysis. Eur J Ophthalmol 2012; 22 Suppl 7:S81-88.
50. Mazzotta C, Traversi C, Caragiuli S, Rechichi M: Pulsed vs continuous light accelerated corneal collagen crosslinking: in vivo qualitative investigation by confocal microscopy and corneal OCT. Eye (Lond) 2014; 28:1179-1183.
51. Bouheraoua N, Jouve L, El Sanharawi M, Sandali O, Temstet C, Loriaut P, Basli E, Borderie V, Laroche L: Optical coherence tomography and confocal microscopy following three different protocols of corneal collagen-crosslinking in keratoconus. Invest Ophthalmol Vis Sci 2014; 55:7601-7609.
52. Wollensak G, Spoerl E, Reber F, Seiler T: Keratocyte cytotoxicity of riboflavin/UVA-treatment in vitro. Eye (Lond) 2004; 18:718-722.
53. Wollensak G, Spoerl E, Wilsch M, Seiler T: Keratocyte apoptosis after corneal collagen cross-linking using riboflavin/UVA treatment. Cornea 2004; 23:43-49.
54. Mazzotta C, Traversi C, Baiocchi S, Caporossi O, Bovone C, Sparano MC, Balestrazzi A, Caporossi A: Corneal healing after riboflavin ultraviolet-A collagen cross-linking determined by confocal laser scanning microscopy in vivo: early and late modifications. Am J Ophthalmol 2008; 146:527-533.
55. Raiskup F, Spoerl E: Corneal cross-linking with hypo-osmolar riboflavin solution in thin keratoconic corneas. Am J Ophthalmol 2011; 152:28-32 e21.
56. O'Brart DP, Chan E, Samaras K, Patel P, Shah SP: A randomised, prospective study to investigate the efficacy of riboflavin/ultraviolet A (370 nm) corneal collagen cross-linkage to halt the progression of keratoconus. Br J Ophthalmol 2011; 95:1519-1524.
57. Vinciguerra P, Albe E, Trazza S, Rosetta P, Vinciguerra R, Seiler T, Epstein D: Refractive, topographic, tomographic, and aberrometric analysis of keratoconic eyes undergoing corneal cross-linking. Ophthalmology 2009; 116:369-378.
58. Magli A, Forte R, Tortori A, Capasso L, Marsico G, Piozzi E: Epithelium-off corneal collagen cross-linking versus transepithelial cross-linking for pediatric keratoconus. Cornea 2013; 32:597-601.
59. Legare ME, Iovieno A, Yeung SN, Kim P, Lichtinger A, Hollands S, Slomovic AR, Rootman DS: Corneal collagen cross-linking using riboflavin and ultraviolet

A for the treatment of mild to moderate keratoconus: 2-year follow-up. Can J Ophthalmol 2013; 48:64-69.

60. Alessio G, L'Abbate M, Furino C, Sborgia C, La Tegola MG: Confocal microscopy analysis of corneal changes after photorefractive keratectomy plus cross-linking for keratoconus: 4-year follow-up. Am J Ophthalmol 2014; 158:476-484.
61. Richoz O, Tabibian D, Hammer A, Majo F, Nicolas M, Hafezi F: The effect of standard and high-fluence corneal cross-linking (CXL) on cornea and limbus. Invest Ophthalmol Vis Sci 2014; 55:5783-5787.
62. Mazzotta C, Caporossi T, Denaro R, Bovone C, Sparano C, Paradiso A, Baiocchi S, Caporossi A: Morphological and functional correlations in riboflavin UV A corneal collagen cross-linking for keratoconus. Acta Ophthalmol 2012; 90:259-265.
63. Thorsrud A, Nicolaissen B, Drolsum L: Corneal collagen crosslinking in vitro: inhibited regeneration of human limbal epithelial cells after riboflavin-ultraviolet-A exposure. J Cataract Refract Surg 2012; 38:1072-1076.
64. Tiffany JM: The normal tear film. Dev Ophthalmol 2008; 41:1-20.
65. Alves M, Fonseca EC, Alves MF, Malki LT, Arruda GV, Reinach PS, Rocha EM: Dry eye disease treatment: a systematic review of published trials and a critical appraisal of therapeutic strategies. Ocul Surf 2013; 11:181-192.
66. Rauz S, Saw VP: Serum eye drops, amniotic membrane and limbal epithelial stem cells-tools in the treatment of ocular surface disease. Cell Tissue Bank 2010; 11:13-27.
67. Fox RI, Chan R, Michelson JB, Belmont JB, Michelson PE: Beneficial effect of artificial tears made with autologous serum in patients with keratoconjunctivitis sicca. Arthritis Rheum 1984; 27:459-461.
68. Tsubota K, Goto E, Shimmura S, Shimazaki J: Treatment of persistent corneal epithelial defect by autologous serum application. Ophthalmology 1999; 106:1984-1989.
69. Noble BA, Loh RSK, MacLennan S, Pesudovs K, Reynolds A, Bridges LR, Burr J, Stewart O, Quereshi S: Comparison of autologous serum eye drops with conventional therapy in a randomised controlled crossover trial for ocular surface disease. Br J Ophthalmol 2004; 88:647-652.
70. Jeng BH, Dupps WJ: Autologous serum 50% eyedrops in the treatment of

persistent corneal epithelial defects. Cornea 2009; 28:1104-1108.

71. Chiang CC, Chen WL, Lin JM, Tsai YY: Allogeneic serum eye drops for the treatment of persistent corneal epithelial defect. Eye 2009; 23:290-293.
72. Harritshoj LH, Nielsen C, Ullum H, Hansen MB, Julian HO: Ready-made allogeneic ABO-specific serum eye drops: production from regular male blood donors, clinical routine, safety and efficacy. Acta Ophthalmologica 2014; 92:783-786.
73. Semeraro F, Forbice E, Braga O, Bova A, Di Salvatore A, Azzolini C: Evaluation of the efficacy of 50% autologous serum eye drops in different ocular surface pathologies. Biomed Res Int 2014; e826970.
74. Geerling G, Daniels JT, Dart JKG, Cree IA, Khaw PT: Toxicity of natural tear substitutes in a fully defined culture model of human corneal epithelial cells. Invest Ophth Vis Sci 2001; 42:948-956.
75. Liu L, Hartwig D, Harloff S, Herminghaus P, Wedel T, Geerling G: An optimised protocol for the production of autologous serum eyedrops. Graef Arch Clin Exp 2005; 243:706-714.
76. Liu L, Hartwig D, Harloff S, Herminghaus P, Wedel B, Kasper K, Geerling G: Corneal epitheliotrophic capacity of three different blood-derived preparations. Invest Ophth Vis Sci 2006; 47:2438-2444.
77. Akyol-Salman I: Effects of autologous serum eye drops on corneal wound healing after superficial keratectomy in rabbits. Cornea 2006; 25:1178-1181.
78. Mukhopadhyay S, Sen S, Datta H: Comparative role of 20% cord blood serum and 20% autologous serum in dry eye associated with Hansen's disease: a tear proteomic study. Brit J Ophthalmol 2015; 99:108-112.
79. Celebi ARC, Ulusoy C, Mirza GE: The efficacy of autologous serum eye drops for severe dry eye syndrome: a randomized double-blind crossover study. Graef Arch Clin Exp 2014; 252:619-626.
80. Urzua CA, Vasquez DH, Huidobro A, Hernandez H, Alfaro J: Randomized double-blind clinical trial of autologous serum versus artificial tears in dry eye syndrome. Curr Eye Res 2012; 37:684-688.
81. Matsuo H, Tomidokoro A, Tomita G, Araie M: Topical application of autologous serum for the treatment of late-onset aqueous oozing or point-leak through. Eye 2005; 19:23-28.
82. Goto E, Shimmura S, Shimazaki J, Tsubota K: Treatment of superior limbic

keratoconjunctivitis by application of autologous serum. Cornea 2001; 20:807-810.

83. Geerling G, MacLennan S, Hartwig D: Autologous serum eye drops for ocular surface disorders. Br J Ophthalmol 2004; 88:1467-1474.
84. Mariappan I, Maddileti S, Savy S, Tiwari S, Gaddipati S, Fatima A, Sangwan VS, Balasubramanian D, Vemuganti GK: In vitro culture and expansion of human limbal epithelial cells. Nat Protoc 2010; 5:1470-1479.
85. Nakamura T, Inatomi T, Sotozono C, Ang LP, Koizumi N, Yokoi N, Kinoshita S: Transplantation of autologous serum-derived cultivated corneal epithelial equivalents for the treatment of severe ocular surface disease. Ophthalmology 2006; 113:1765-1772.
86. Nakamura T, Ang LPK, Rigby H, Sekiyama E, Inatomi T, Sotozono C, Fullwood NJ, Kinoshita S: The use of autologous serum in the development of corneal and oral epithelial equivalents in patients with Stevens-Johnson syndrome. Invest Ophth Vis Sci 2006; 47:909-916.
87. Koizumi N, Inatomi T, Sotozono C, Fullwood NJ, Quantock AJ, Kinoshita S: Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res 2000; 20:173-177.
88. Lim LS, Riau A, Poh R, Tan DT, Beuerman RW, Mehta JS: Effect of dispase denudation on amniotic membrane. Mol Vis 2009; 15:1962-1970.
89. Kniss DA, Zimmerman PD, Su HC, Hooper WC, Landon MB, Gabbe SG: Expression of functional insulin-like growth factor-i receptors by human amnion cells. Am J Obstet Gynecol 1993; 169:632-640.
90. Mamede AC, Carvalho MJ, Abrantes AM, Laranjo M, Maia CJ, Botelho MF: Amniotic membrane: from structure and functions to clinical applications. Cell Tissue Res 2012; 349:447-458.
91. Fukuda K, Chikama T, Nakamura M, Nishida T: Differential distribution of subchains of the basement membrane components type IV collagen and laminin among the amniotic membrane, cornea, and conjunctiva. Cornea 1999; 18:73-79.
92. Kubo M, Sonoda Y, Muramatsu R, Usui M: Immunogenicity of human amniotic membrane in experimental xenotransplantation. Invest Ophth Vis Sci 2001; 42:1539-1546.
93. Sorsby A, Symons HM: Amniotic membrane grafts in caustic burns of the eye (burns of the second degree). Br J Ophthalmol 1946; 30:337-345.

94. Kim JC, Tseng SC: Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea 1995; 14:473-484.

95. Seitz B, Resch MD, Schlötzer-Schrehardt U, Hofmann-Rummelt C, Sauer R, Kruse FE: Histopathology and ultrastructure of human corneas after amniotic membrane transplantation. Arch Ophthalmol 2006; 124:1487-1490.

96. Jiang A, Li C, Gao Y, Zhang M, Hu J, Kuang W, Hao S, Yang W, Xu C, Gao G, Wang Z, Liu Z: In vivo and in vitro inhibitory effect of amniotic extraction on neovascularization. Cornea 2006; 25(10 Suppl 1):S36-40.

97. Liang L, Li W, Ling S, Sheha H, Qiu W, Li C, Liu Z: Amniotic membrane extraction solution for ocular chemical burns. Clin Experiment Ophthalmol 2009; 37:855-863.

98. Choi JA, Jin HJ, Jung S, Yang E, Choi JS, Chung SH, Joo CK: Effects of amniotic membrane suspension in human corneal wound healing in vitro. Mol Vis 2009; 15:2230-2238.

99. Kobayashi N, Kabuyama Y, Sasaki S, Kato K, Homma Y: Suppression of corneal neovascularization by culture supernatant of human amniotic cells. Cornea 2002; 21:62-67.

100. Kamiya K, Wang M, Uchida S, Amano S, Oshika T, Sakuragawa N, Hori J: Topical application of culture supernatant from human amniotic epithelial cells suppresses inflammatory reactions in cornea. Exp Eye Res 2005; 80:671-679.

101. Wichayacoop T, Briksawan P, Tuntivanich P, Yibchok-Anun S: Anti-inflammatory effects of topical supernatant from human amniotic membrane cell culture on canine deep corneal ulcer after human amniotic membrane transplantation. Vet Ophthalmol 2009; 12:28-35.

102. Bonci P, Bonci P, Lia A: Suspension made with amniotic membrane: Clinical trial. Eur J Ophthalmol 2005; 15:441-445.

103. Kordic R, Suic SP, Jandrokovic S, Kalauz M, Kuzman T, Skegro I, Jukic T: Application of the amniotic membrane extract (AMX) for the persistent epithelial defect (PED) of the cornea. Collegium Antropol 2013; 37:161-164.

104. Guo Q, Hao J, Yang Q, Guan LN, Ouyang S, Wang JW: A comparison of the effectiveness between amniotic membrane homogenate and transplanted amniotic membrane in healing corneal damage in a rabbit model. Acta Ophthalmol 2011; 89:E315-E319.

105. Seitz B, Hayashi SJ, Wee WR, LaBree L, McDonnell PJ: In vitro effects of aminoglycosides and fluoroquinolones on keratocytes. Invest Ophth Vis Sci 1996; 37:656-665.
106. Song XF, Stachon T, Wang J, Langenbucher A, Seitz B, Szentmáry N: Viability, apoptosis, proliferation, activation and cytokine secretion of human keratoconus keratocytes after crosslinking. Biomed Res Int 2015; e254237.
107. Cadet J, Mouret S, Ravanat JL, Douki T: Photoinduced damage to cellular DNA: direct and photosensitized reactions. Photochem Photobiol 2012; 88:1048-1065.
108. Kymionis GD, Portaliou DM, Bouzoukis DI, Suh LH, Pallikaris AI, Markomanolakis M, Yoo SH: Herpetic keratitis with iritis after corneal crosslinking with riboflavin and ultraviolet A for keratoconus. J Cataract Refract Surg 2007; 33:1982-1984.
109. Kymionis GD, Bouzoukis DI, Diakonis VF, Portaliou DM, Pallikaris AI, Yoo SH: Diffuse lamellar keratitis after corneal crosslinking in a patient with post-laser in situ keratomileusis corneal ectasia. J Cataract Refract Surg 2007; 33:2135-2137.
110. Rama P, Di Matteo F, Matuska S, Paganoni G, Spinelli A: Acanthamoeba keratitis with perforation after corneal crosslinking and bandage contact lens use. J Cataract Refr Surg 2009; 35:788-791.
111. Kim WJ, Rabinowitz YS, Meisler DM, Wilson SE: Keratocyte apoptosis associated with keratoconus. Exp Eye Res 1999; 69:475-481.
112. Mace M, Galiacy SD, Erraud A, Mejia JE, Etchevers H, Allouche M, Desjardins L, Calvas P, Malecaze F: Comparative transcriptome and network biology analyses demonstrate antiproliferative and hyperapoptotic phenotypes in human keratoconus corneas. Invest Ophthalmol Vis Sci 2011; 52:6181-6191.
113. Iqbal O, Fisher G, Vira S, Syed D, Sadeghi N, Freeman D, Campbell E, Sugar J, Feder R, Fareed J, Bouchard C: Increased expression of secreted frizzled-related protein-1 and microtubule-associated protein light chain 3 in keratoconus. Cornea 2013; 32:702-707.
114. Matthews FJ, Cook SD, Majid MA, Dick AD, Smith VA: Changes in the balance of the tissue inhibitor of matrix metalloproteinases (TIMPs)-1 and-3 may promote keratocyte apoptosis in keratoconus. Exp Eye Res 2007; 84:1125-1134.
115. Collier SA, Madigan MC, Penfold PT: Expression of membrane-type 1 matrix metalloproteinase (MT1-MMP) and MMP-2 in normal and keratoconus corneas. Curr Eye Res 2000; 21:662-668.

116. Chwa M, Atilano SR, Reddy V, Jordan N, Kim DW, Kenney MC: Increased stress-induced generation of reactive oxygen species and apoptosis in human keratoconus fibroblasts. Invest Ophthalmol Vis Sci 2006; 47:1902-1910.
117. Mallet JD, Rochette PJ: Wavelength-dependent ultraviolet induction of cyclobutane pyrimidine dimers in the human cornea. Photochem Photobiol Sci 2013; 12:1310-1318.
118. Rouhi L, Kajbafzadeh AM, Modaresi M, Shariati M, Hamrahi D: Autologous serum enhances cardiomyocyte differentiation of rat bone marrow mesenchymal stem cells in the presence of transforming growth factor-beta1 (TGF-beta1). In Vitro Cell Dev Biol Anim 2013; 49:287-294.
119. Bahn JJ, Chung JY, Im W, Kim M, Kim SH: Suitability of autologous serum for expanding rabbit adipose-derived stem cell populations. J Vet Sci 2012; 13:413-417.
120. Morimoto N, Takemoto S, Kanda N, Ayvazyan A, Taira MT, Suzuki S: The utilization of animal product-free media and autologous serum in an autologous dermal substitute culture. J Surg Res 2011; 171:339-346.
121. Takeda A, Yamazaki Y, Baba K, Ishiguro M, Aoyagi K, Ikemoto S, Uchinuma E: Osteogenic potential of human bone marrow-derived mesenchymal stromal cells cultured in autologous serum: a preliminary study. J Oral Maxillofac Surg 2012; 70:e469-476.
122. Josh F, Kobe K, Tobita M, Tanaka R, Suzuki K, Ono K, Hyakusoku H, Mizuno H: Accelerated and safe proliferation of human adipose-derived stem cells in medium supplemented with human serum. J Nippon Med Sch 2012; 79:444-452.
123. Shumiya T, Shibata R, Shimizu Y, Ishii M, Kubota R, Shintani S, Murohara T: Evidence for the therapeutic potential of ex vivo expanded human endothelial progenitor cells using autologous serum. Circ J 2010; 74:1006-1013.
124. Yan L, Wu W, Wang Z, Li C, Lu X, Duan H, Zhou J, Wang X, Wan P, Song Y, Tang J, Han Y: Comparative study of the effects of recombinant human epidermal growth factor and basic fibroblast growth factor on corneal epithelial wound healing and neovascularization in vivo and in vitro. Ophthalmic Res 2013; 49:150-160.
125. Zhang JL, Upadhya D, Lu L, Reneker LW: Fibroblast growth factor receptor 2 (FGFR2) is required for corneal epithelial cell proliferation and differentiation during embryonic development. Plos One 2015; 10: e0117089.

126. Zelenka PS, Arpitha P: Coordinating cell proliferation and migration in the lens and cornea. Semin Cell Dev Biol 2008; 19:113-124.
127. Raeder S, Utheim TP, Messelt E, Lyberg T: The impact of de-epithelialization of the amniotic membrane matrix on morphology of cultured human limbal epithelial cells subject to eye bank storage. Cornea 2010; 29:439-445.
128. Shortt AJ, Secker GA, Lomas RJ, Wilshaw SP, Kearney JN, Tuft SJ, Daniels JT: The effect of amniotic membrane preparation method on its ability to serve as a substrate for the ex-vivo expansion of limbal epithelial cells. Biomaterials 2009; 30:1056-1065.
129. Chen B, Mi SL, Wright B, Connon CJ: Differentiation status of limbal epithelial cells cultured on intact and denuded amniotic membrane before and after air-lifting. Tissue Eng Part A 2010; 16:2721-2729.
130. Koizumi N, Rigby H, Fullwood NJ, Kawasaki S, Tanioka H, Koizumi K, Kociok N, Joussen AM, Kinoshita S: Comparison of intact and denuded amniotic membrane as a substrate for cell-suspension culture of human limbal epithelial cells. Graef Arch Clin Exp 2007; 245:123-134.
131. Ahmadiankia N, Ebrahimi M, Hosseini A, Baharvand H: Effects of different extracellular matrices and co-cultures on human limbal stem cell expansion in vitro. Cell Biol Int 2009; 33:978-987.
132. Galindo EEH, Theiss C, Pauklin M, Wettey FR, Steuhl KP, Meller D: Correlation of cell cycle kinetics with p63 expression in human limbal epithelial cells expanded on intact human amniotic membrane. Ophthal Res 2009; 41:83-90.
133. Yam HF, Pang CP, Fan DSP, Fan BJ, Yu EYW, Lam DSC: Growth factor changes in ex vivo expansion of human limbal epithelial cells on human amniotic membrane. Cornea 2002; 21:101-105.
134. Lee JH, Ryu IH, Kim EK, Lee JE, Hong S, Lee HK: Induced expression of insulin-like growth factor-1 by amniotic membrane-conditioned medium in cultured human corneal epithelial cells. Invest Ophth Vis Sci 2006; 47:864-872.
135. Ma DHK, Lai JY, Yu ST, Liu JY, Yang U, Chen HCJ, Yeh LK, Ho YJ, Chang G, Wang SF et al: Up-regulation of heat shock protein 70-1 (Hsp70-1) in human limbo-corneal epithelial cells cultivated on amniotic membrane: A proteomic study. Journal of Cellular Physiology 2012; 227:2030-2039.
136. Konomi K, Satake Y, Shimmura S, Tsubota K, Shimazaki J: Long-term results of amniotic membrane transplantation for partial limbal deficiency. Cornea 2013;

32:1110-1115.

137. Berguiga M, Mameletzi E, Nicolas M, Rivier D, Majo F: Long-term follow-up of multilayer amniotic membrane transplantation (MLAMT) for non-traumatic corneal perforations or deep ulcers with descemetocele. Klin Monatsbl Augenh 2013; 230:413-418.
138. Gris O, Lopez-Navidad A, Caballero F, del Campo Z, Adan A: Amniotic membrane transplantation for ocular surface pathology: Long-term results. Transplant Proc 2003; 35:2031-2035.
139. Shahriari HA, Tokhmehchi F, Reza M, Hashemi NF: Comparison of the effect of amniotic membrane suspension and autologous serum on alkaline corneal epithelial wound healing in the rabbit model. Cornea 2008; 27:1148-1150.
140. Sakuragawa N, Elwan MA, Uchida S, Fujii T, Kawashima K: Non-neuronal neurotransmitters and neurotrophic factors in amniotic epithelial cells: Expression and function in humans and monkey. Jpn J Pharmacol 2001; 85:20-23.
141. Uchida S, Suzuki Y, Araie M, Kashiwagi K, Otori Y, Sakuragawa N: Factors secreted by human amniotic epithelial cells promote the survival of rat retinal ganglion cells. Neurosci Lett 2003; 341:1-4.
142. Shao CK, Sima J, Zhang SX, Jin J, Reinach P, Wang Z, Ma JX: Suppression of corneal neovascularization by PEDF release from human amniotic membranes. Invest Ophth Vis Sci 2004; 45:1758-1762.
143. Zhang T, Yam GHF, Riau AK, Poh R, Allen JC, Peh GS, Beuerman RW, Tan DT, Mehta JS: The effect of amniotic membrane de-epithelialization method on its biological properties and ability to promote limbal epithelial cell culture. Invest Ophth Vis Sci 2013; 54:3072-3081.
144. Tan YH, Qiu FF, Qu YL, Li C, Shao Y, Xiao QG, Liu ZG, Li W: Amniotic membrane inhibits squamous metaplasia of human conjunctival epithelium. Am J Physiol Cell Physiol 2011; 301:C115-C125.
145. Li HC, Niederkorn JY, Neelam S, Mayhew E, Word RA, McCulley JP, Alizadeh H: Immunosuppressive factors secreted by human amniotic epithelial cells. Invest Ophth Vis Sci 2005; 46:900-907.
146. Wolbank S, Peterbauer A, Fahrner M, Hennerbichler S, Van Griensven M, Stadler G, Redl H, Gabriel C: Dose-dependent immunomodulatory effect of human stem cells from amniotic membrane: A comparison with human mesenchymal stem

cells from adipose tissue. Tissue Eng 2007; 13:1173-1183.

147. Jiao HL, Guan FX, Yang B, Li JB, Song LJ, Hu X, Du Y: Human amniotic membrane derived-mesenchymal stem cells induce C6 glioma apoptosis in vivo through the Bcl-2/caspase pathways. Mol Biol Rep 2012; 39:467-473.
148. Kang NH, Yi BR, Lim SY, Hwang KA, Baek YS, Kang KS, Choi KC: Human amniotic membrane-derived epithelial stem cells display anticancer activity in BALB/c female nude mice bearing disseminated breast cancer xenografts. Int J Oncol 2012; 40:2022-2028.
149. Mamede AC, Laranjo M, Carvalho MJ, Abrantes AM, Pires AS, Brito AF, Moura P, Maia CJ, Botelho MF: Effect of amniotic membrane proteins in human cancer cell lines: an exploratory study. J Membrane Biol 2014; 247:357-360.
150. Magatti M, De Munari S, Vertua E, Parolini O: Amniotic membrane-derived cells inhibit proliferation of cancer cell lines by inducing cell cycle arrest. J Cell Mol Med 2012; 16:2208-2218.

ACKNOWLEDGEMENTS

First of all I want to express my sincere gratitude to Prof. Seitz, who introduced me into the MD/PhD program of Saarland University and gave me meticulous supervision, offered me the chance to participate these interesting topics.

Many thanks to Dr. Szentmáry, my “Doktormutter”, who planned my doctoral topics and corrected countless documents for my paperwork. I could never finish my dissertation without her generous help.

Thanks to my helpful colleague Ms. Stachon for all support at the biological lab of the Department of Ophthalmology and for the help in translation of the summary of this dissertation to german language.

Thanks to my other colleagues, Dr. Wang and Dr. Song, who helped me a lot not only in my research but also in the daily life, and also Dr. Colanesi, my very nice friend.

Thanks to Prof. Langenbucher, from whom I had learnt a lot about statistics. And also thanks to Prof. Wagenpfeil, who introduced us Generalized Estimating Equations.

Thanks to Prof. Tschernig, PD Dr. Bischoff and Ms. Deichelbohrer, with whom I had worked on another wonderful topic, which is not included in this dissertation.

There are so many people I would like to thank to, although I have just stayed in Europe, mostly in Homburg, for three years, I have met people willing to help everywhere. But I have to prevent the acknowledgements from becoming the longest section of my dissertation.

Now, above all, thanks to my parents who raised me up and sponsored my study of medicine for ten years, they are also the main reason why I study medicine. I wouldn’t have chosen this tough way if they didn’t insist. I am proud, since I know I really make them proud.

I deeply appreciate my Chinese friends at the other edge of the continent, with whom I could have some daily small talk via WeChat, they have helped me to survive the profound loneliness in this small town.

Finally, thank goodness, I am going back to China and working as a respected physician!

PUBLICATIONS

[1] Ming-Feng Wu, Tanja Stachon, Jiong Wang, Xuefei Song, Sarah Colanesi, Berthold Seitz, Stefan Wagenpfeil, Achim Langenbucher, Nóra Szentmáry. Effect of keratocyte supernatant on epithelial cell migration and proliferation after corneal crosslinking (CXL). Curr Eye Res. 2015 Aug 3:1-8. [Epub ahead of print]

[2] Ming-Feng Wu, Tanja Stachon, Achim Langenbucher, Berthold Seitz, Nóra Szentmáry. Effect of amniotic membrane suspension (AMS) and amniotic membrane homogenate (AMH) on human corneal epithelial cell viability, migration and proliferation in vitro. Curr Eye Res. [Accepted]

[3] Ming-Feng Wu, Tanja Stachon, Berthold Seitz, Achim Langenbucher, Nóra Szentmáry. Effect of human autologous serum (AS) and fetal bovine serum (FBS) on human corneal epithelial cell viability, migration and proliferation in vitro. Int J Ophthalmol. [Submitted]

[4] Ming-Feng Wu, Mona Deichelbohrer, Thomas Tschernig, Matthias W. Laschke, Nóra Szentmáry, Dirk Hüttenberger, Hans-Jochen Foth, Berthold Seitz, Markus Bischoff. Chlorin e6 mediated photodynamic inactivation for multidrug resistant *Pseudomonas aeruginosa* keratitis in mice in vivo. [Under preparation].

CURRICULUM VITAE

Personal data:

Name:	Ming-Feng Wu
Sex:	Male
Date and place of birth:	31 May, 1988, in Guangxi Zhuang Autonomous Region
Nationality	People's Republic of China (P.R. China)
Address:	Klinik für Augenheilkunde und Hochschulambulanz
	Universitätsklinikum des Saarlandes UKS
	Kirrbergerstrasse 100
	D-66424 Homburg/Saar
	Tel.: +49-6841-1622325
	Fax: +49-6841-1622400
E-mail:	enmengfong@hotmail.com

Education:

09. 2000 – 06. 2006	Pubei Middle and High School, Guangxi, P.R. China
09. 2006 – 06. 2013	Medical School, Tongji University, Shanghai, P.R. China
10. 2013 –	MD/PhD program, Department of Ophthalmology, Saarland University Medical Center, Homburg/Saar, Germany