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Pathogenese des Keratokonus – inflammatorische Komponente durch metabolische Veränderungen?

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1. Zusammenfassung

Der Keratokonus (KC) ist eine progressive Erkrankung der Hornhaut. Beim Keratokonus wird die Hornhaut dünner und wölbt sich konisch nach vorne. Die Erkrankung tritt in der Regel im Teenageralter auf. Etwa 1 von 2000 aus der Bevölkerung sind in Europa von der Erkrankung betroffen. Bei einem Teil der Patienten verlangsamt sich die Erkrankung oder die Progression stoppt, bei einem Teil schreitet die Progression immer weiter. Die Behandlung erfolgt zunächst mit harten Kontaktlinsen, um die nachlassende Sehschärfe auszugleichen. Für einige Patienten kommen das Riboflavin-Crosslinking oder die Implantation intrakornealer Ringsegmente als Behandlungsmethode in Frage, um die Progression der Erkrankung zu stoppen. Für einen Teil der Patienten bleibt nur noch die Hornhauttransplantation, um die Sehschärfe zu verbessern.

In der ersten Arbeit wurden Harnstoff, Harnsäure, Prolaktin und fT4 im Kammerwasser von Keratokonuspatienten untersucht. Es konnte gezeigt werden, dass die Harnstoff und Prolaktin Konzentrationen im Kammerwasser von KC-Patienten erniedrigt sind, während fT4 erhöht ist. Da der Harnstoffzyklus bei der Biosynthese von Aminosäuren im Sinne der Kollagensynthese eine entscheidende Rolle spielt, wurde dieser Parameter als Ausgangspunkt für weitere Studien angesehen.

In der zweiten Studie wurde in Zellkulturversuchen gezeigt, dass die Harnstoff Konzentration auch in den Keratozyten von KC-Patienten reduziert ist. Da Harnstoff ein Nebenprodukt bei der Synthese von Prolin und Hydroxyprolin ist, haben wir den Harnstoffzyklus in den Keratozyten genauer untersucht. Die Ergebnisse dieser Arbeit lieferten weitere Hinweise auf eine veränderte Regulation der Kollagensynthese in KC-Keratozyten. Die Studie ergab, dass die Keratokonus Keratozyten eine erniedrigte Arginase Aktivität und eine erniedrigte Hydroxyprolin Konzentration im Vergleich zu den gesunden Kontrollzellen aufweisen.

Die Ergebnisse der dritten Arbeit unterstützen die These einiger Forschungsgruppen, dass die Krankheit mit entzündlichen Prozessen einhergeht. Ein Schlüssel-Gen bei entzündlichen Prozessen ist der Nuklear Faktor kappa B (NF-κB). Als Transkriptionsfaktor aktiviert NF-κB unter anderem das bei entzündlichen Prozessen mitwirkende Enzym Nitric Oxide Synthase (NOS). Die Ergebnisse dieser Arbeit zeigten eine erhöhte NF-κB mRNA und Protein Expression, sowie auch eine erhöhte Stickoxidproduktion und induzierte NOS mRNA Expression in Keratokonus Keratozyten.

Weitere darauf aufbauende Untersuchungen sind notwendig, um mehr Einblick in die Erkrankung und damit auch die Möglichkeit neuer Behandlungsmethoden für die Patienten durch weitere Erkenntnisse hinsichtlich der Pathogenese zu gewinnen.

Summary

Pathogenesis of Keratoconus – Inflammatory components through metabolic changes?

Keratoconus (KC) is a progressive corneal disease, which is characterized by corneal thinning and conical shape. It usually appears in teenagers and about 1 of 2000 persons is affected in Europe. In some of the patients progression is getting slower with age and then it stops, in some others it accelerates. The stage-related treatment starts with rigid gas permeable contact lenses to compensate corneal irregularities and to improve vision. In case of progression, riboflavin crosslinking or implantation of intracorneal ring segments may be used to stabilize the cornea. In some of the patients, corneal transplantation is performed in order to improve visual acuity.

In the first paper urea, uric acid, prolactin and fT4 concentration were examined in aqueous humor of keratoconus patients. It could be shown that urea and prolactin concentrations are decreased and fT4 concentration is increased in aqueous humor of keratoconus patients. Since the urea cycle plays a decisive role in the biosynthesis of non-essential proteinogenic amino acids in terms of collagen synthesis, this parameter was considered as a crucial starting point for further studies.

In the second study, a reduced urea concentration could be verified in keratoconus keratocyte cultures. Since urea is a by-product in the synthesis of proline and hydroxyproline, we examined the urea cycle in keratocytes more closely. The results of this work provided further evidence for altered regulation of collagen synthesis in KC-keratocytes. Here, a decreased arginase activity and hydroxyproline concentration could be shown in keratoconus keratocytes, compared to normal controls.

The results of the third study supported the hypothesis of some research groups, that keratoconus is associated with inflammatory processes. A key gene in inflammatory processes is the nuclear factor kappa B (NF-κB). As a transcription factor, NF-κB activates the enzyme Nitric Oxide Synthase (NOS), which is involved in inflammatory processes. The results of this work showed an increased NF-κB mRNA and protein expression, as well as increased nitric oxide production and induced NOS mRNA expression in keratoconus keratocytes.

Further investigations based on these results are necessary in order to gain more insight into the pathogenesis of keratoconus and thus, to new treatment modalities for the patients.

2. Einleitung und Motivation

Der Keratokonus (KC) ist eine schwerwiegende Erkrankung der Hornhaut. Die Hornhaut wird dünner und aufgrund der nachlassenden Stabilität wölbt sie sich konisch nach vorne, wodurch sich die Sehschärfe verschlechtert (**Abbildung 1**). Der Keratokonus ist eine Erkrankung, die nach neueren Erkenntnissen die üblicherweise zitierte Prävalenz von 1/2000 deutlich überschreitet und mit einer bis zu 10-fach erhöhten Zahl (265 Fälle pro 100 000) angegeben wird [20]. Die Erkrankung tritt in der Regel im Teenageralter auf, das Durchschnittsalter der Diagnose in Europa liegt bei etwa 28 Jahren, davon sind ca. 60% Männer [20]. In der Regel tritt die Erkrankung an beiden Augen auf, jedoch meist in zeitlichem Abstand und in unterschiedlicher Ausprägung. Bei manchen Patienten schreitet die Erkrankung nicht weiter fort, bei anderen jedoch entwickelt sich ein schwerwiegender Verlauf.

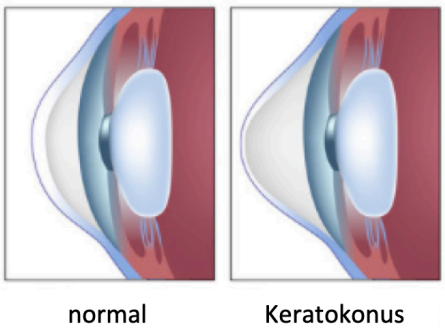

Abbildung 1. Keratokonus
Die gesunde Hornhaut ist gleichmäßig gewölbt, während beim Keratokonus die Hornhaut sichtbar dünner und konisch nach vorne gewölbt ist. Quelle: http://www.nseye.com/complete-eye-care/about-the-cornea/corneal-conditions/keratoconus

Die Hornhaut stellt als vorderste Schicht des Auges gleichsam die „Windschutzscheibe“ dar (**Abbildung 2**). Das mehrschichtige Epithel schützt die Kornea vor mechanischer Beanspruchung und ist die erste Barriere bei der Immunreaktion. Die zweite Schicht ist das Stroma, in das die Keratozyten eingebettet sind. Diese Zellen produzieren die extrazelluläre Matrix inklusive Kollagene, hauptsächlich Typ I Kollagen (80-90%) und Typ V Kollagen. Die Endothelzellen sorgen für die Erhaltung der Transparenz der Hornhaut, indem sie für einen ausgeglichenen Hydratationszustand sorgen.

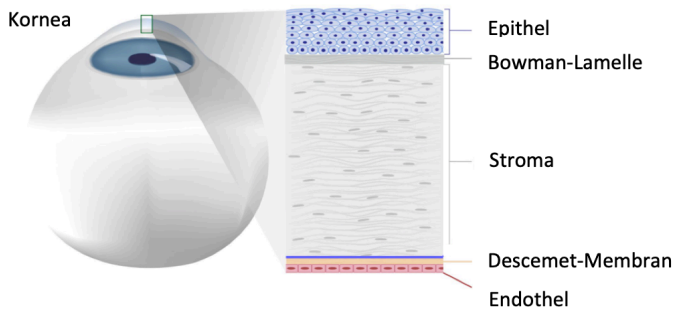

Abbildung 2. Die Hornhaut
Der Querschnitt zeigt die Hornhaut mit den 3 Zellschichten, getrennt durch Bowman-Lamelle und Descemet-Membran. Das mehrschichtige Epithel ist ca. 50µm dick, das Stroma bei einer gesunden Kornea ca. 500µm. Das Endothel ist einschichtig und in der Regel ca. 5µm dick. Quelle: http://www.konanmedical.com/cellcheck/specular-fundamentals/

Die Behandlung des Keratokonus läuft in der Regel stadiengerecht ab. Im frühen Stadium der Erkrankung können die Patienten die nachlassende Sehschärfe zunächst mit harten Kontaktlinsen ausgleichen. Ist die Verdünnung der Hornhaut noch nicht zu weit fortgeschritten, können dem Patienten Intrakorneale Ringsegmente (INTACS) eingesetzt werden, um die ungleichmäßige Verkrümmung auszugleichen. Dadurch steigt der unkorrigierte und der korrigierte Visus und die Patienten können wieder besser Kontaktlinsen tragen, um die Sehschärfe weiter zu verbessern. INTACS können die Hornhaut auch stabilisieren und möglicherweise zu einer Verlangsamung oder zu einem Stopp der Progression führen. Bei einem Teil der Patienten mit progressivem Befund kann das sogenannte Riboflavin-Crosslinking durchgeführt werden. Hierbei wird den Patienten Riboflavin auf die deepithelialisierte Hornhaut getropft und anschließend mit UVA-Licht bestrahlt, was bei einem Großteil der Patienten zu einer Stabilisierung der Hornhaut durch eine Quervernetzung der Kollagenfasern führt. Wenn die korrigierte Sehschärfe für den Patienten unzureichend ist, kann eine Hornhauttransplantation indiziert sein, um die Sehschärfe zu verbessern.

Die Ursachen des Keratokonus sind bisher weitgehend ungeklärt, jedoch werden genetische Ursachen und biochemische Veränderungen diskutiert. Die veränderten Gene stehen im Zusammenhang mit der Kollagensynthese (COL1A1 und COL5A1) und der Kollagenstabilität (LOX) [1]. Insgesamt werden bis heute etwa 20 Kandidaten-Gene mit dem Keratokonus in Verbindung gebracht. In „genome-wide association studies“ (GWAS), genome-wide linkage studies (GWLS) und „whole exome sequencing“ (WES) wurden in der Regel Punktmutationen beobachtet, die eine Korrelation zur Erkrankung zeigen. Allerdings konnte in keiner dieser Studien bisher ein funktioneller Zusammenhang nachgewiesen werden, weshalb man bisher noch nicht von

einer genetisch bedingten Erkrankung sprechen kann, obwohl familiäre Häufungen beobachtet wurden.

Die Hornhaut verliert jedoch nicht nur ihre Stabilität, sondern wird im Verlauf auch dünner. Die Degradation der Kollagenschicht des Stromas wird mit einem erhöhten Anteil an proteolytischen Enzymen und Matrix-Metalloproteinasen (MMP's) in Verbindung gebracht [4, 14].

Bei Patienten mit einem Keratokonus ist außerdem ein erhöhter oxidativer Stress-Level nachgewiesen worden, assoziiert mit einem erhöhten Status an Antioxidantien. So sind die Antioxidantien Tyrosin und Harnsäure in der Tränenflüssigkeit ebenso erhöht wie die reaktiven Sauerstoffspezies ROS [12].

Der Keratokonus geht häufig mit einem trockenen Auge einher [66]. In der Studie von Jäger et al. wird in diesem Zusammenhang von einem erniedrigten Harnstoffwert in Tränenflüssigkeit bei Patienten mit trockenem Auge berichtet. Hormonelle Ursachen werden ebenfalls in Verbindung mit dem Keratokonus gebracht. So sind bei Patienten mit Keratokonus erniedrigte Estron Konzentrationen und erhöhte DHEA Werte gemessen worden [24, 40]. Ebenso konnte in humanen Zellkulturen von Knorpelzellen eine Zunahme der Kollagen 1 Konzentration nach Thyroxin Gabe (in Kombination mit Ascorbinsäure) beobachtet werden [6].

Bisher galt der Keratokonus als nicht-entzündliche Erkrankung. Jedoch gibt es immer mehr Hinweise, dass die Erkrankung doch mit einer milden subklinischen Entzündung verknüpft sein könnte, auch wenn nicht alle Kriterien einer Entzündung vorliegen. So wurden in der Tränenflüssigkeit von Patienten mit Keratokonus erhöhte Werte von Interleukin (IL) IL-4, IL-5, IL-6, IL-8, TNF-α, und TNF-β nachgewiesen, die in der Regel bei entzündlichen Prozessen vermehrt ausgeschüttet werden [1, 27, 36].

Karamichos et al. haben gezeigt, dass in Tränenflüssigkeit und in kornealen Fibroblasten von Keratokonus-Patienten die Stoffwechselwege in Bezug auf Oxidationszustand, Zitronensäurezyklus sowie den Arginin-Metabolismus im Vergleich zu normalen Kontrollen signifikant verändert sind [28].

Immer wieder werden auch mechanische Ursachen wie das Augenreiben als Auslöser der Erkrankung diskutiert. Gatinel zeigt in seiner Studie, dass ein Großteil seiner Patienten mit einem Keratokonus dauerhaft auf dem Bauch schläft, und ein Auge auf dem Unterarm liegend „komprimiert" wird. Er vertritt daher die Ansicht, der Keratokonus sei eine unilaterale Erkrankung. Dieser mechanische Druck auf ein Auge führe dann zu einem unangenehmen Gefühl bei Tag, so dass die Patienten ein übermäßiges Bedürfnis haben, die Augen zu reiben [17].

Bisher wurde schon eine Vielzahl an pathologischen Veränderungen in den Epithelzellen und den stromalen Zellen der Hornhaut sowie in der Tränenflüssigkeit bei Patienten mit Keratokonus

nachgewiesen. Die übergeordneten Bereiche, metabolische Fehlfunktionen, entzündliche Komponenten und Veränderungen im oxidativen Status beeinflussen sich gegenseitig. So gehen entzündliche Prozesse in der Regel mit einem erhöhten oxidativen Stresslevel einher, genauso wie metabolische Fehlfunktionen dazu führen können, dass entzündliche Prozesse ausgelöst werden. Hormone werden wiederum für einen reibungslosen Ablauf metabolischer Funktionen benötigt.

Ein besseres Verständnis des Keratokonus ist notwendig, um die Behandlungsmethoden und möglicherweise auch eine Prävention der Erkrankung zu erreichen.

In unseren Arbeiten wollten wir systematisch den Fokus auf den Zusammenhang der Komponenten metabolische Funktionen, oxidativer Stresslevel und entzündliche Einflüsse lenken.

Um einen Überblick zu erhalten, untersuchten wir daher in einer größeren Studie das Kammerwasser von Patienten mit einem Keratokonus auf einige Schlüsselparameter hinsichtlich der Kollagensynthese, Antioxidantien und Hormonlevel. Die Ergebnisse aus dieser Studie lieferten den Grundstock für die zweite Arbeit, in der wir zunächst Teile der Kollagensynthese beleuchtet haben, um in der dritten Arbeit einen Einblick über potentielle entzündliche Ursachen zu erhalten.

Harnstoff, Harnsäure, Prolaktin und fT4 Konzentrationen im Kammerwasser von Patienten mit Keratokonus (Veröffentlichung 1)

Diese Studie wurde von der Ethikkommission der Ärztekammer Saarland und Rheinlandpfalz (Nr. 56/14) genehmigt. Für die erste Veröffentlichung wurden Kammerwasserproben von 100 Patienten mit einem diagnostizierten Keratokonus und von 100 Patienten mit Katarakt als Kontrollgruppe die Parameter Harnstoff, Harnsäure, Prolaktin und fT4 gemessen. Da das Probenvolumen von Kammerwasserproben in der Regel sehr klein ist (maximal 50 µl), fand die Probenmessung in Kooperation mit dem Westpfalz-Klinikum in Kaiserslautern statt. Die Analysegeräte des Westpfalz-Klinikums sind auf sehr geringe Probenvolumina ausgerichtet (ADVIA 1800 Chemistry System und ADVIA Centaur XPT Immunoassay System von Siemens Healthcare Diagnostik GmbH, Erlangen, Deutschland). Um die Effekte von Alter, Geschlecht und Diagnose auf die Ergebnisse auszuschließen, wurde zur Berechnung der statistischen Analyse ein generalisiertes lineares Modell (GLM) verwendet.

Die statistische Analyse zeigte, dass bei den Patienten mit Keratokonus die Harnstoffkonzentration erniedrigt ist, unabhängig von Alter und Geschlecht. Es konnte gezeigt werden, dass die Harnstoff und Prolaktin Konzentrationen im Kammerwasser von KC-Patienten erniedrigt sind, während fT4 erhöht ist. Dieses Ergebnis wurde in der Folgestudie in Zellkulturversuchen mit Analysen an Keratozyten von Patienten mit einem Keratokonus weiter verifiziert.

Arginase Aktivität, Harnstoff und Hydroxyprolin Konzentrationen in Keratokonus Keratozyten (Veröffentlichung 2)

Aufbauend auf den Ergebnissen der ersten Publikation wurden in dieser Studie primäre Keratozyten von Patienten mit Keratokonus und Keratozyten von Hornhautspendern, welche nicht den Kriterien zur Transplantation entsprachen, untersucht (Nr. der Ethikkommission 263/15). Hier stellte sich die Frage, ob sich die erniedrigten Harnstoffkonzentrationen aus dem Kammerwasser auch in den Keratozyten messen lassen, oder ob weitere Ursachen für den erniedrigten Wert in Frage kommen.

Es wurde gezeigt, dass die Harnstoff Konzentration der KC-Keratozyten auch in der Zellkultur reduziert ist. Harnstoff entsteht hier als Nebenprodukt bei der Umwandlung von L-Arginin zu L-Ornithin mithilfe des Enzyms *Arginase*. Wenn eine Störung in diesem Schritt auftritt, könnte dies zu verminderten Harnstoffkonzentrationen führen.

Die Arginase-Aktivität zeigte in unseren Versuchen eine verminderte Aktivität in den Keratozyten von KC-Patienten. Harnstoff ist nicht nur ein Nebenprodukt in der Aminosäuresynthese, sondern zeigt auch proliferative Effekte. Die Gruppe um Möslinger konnte dies in Studien mit Makrophagen zeigen [43].

Im weiteren Verlauf wird L-Ornithin zu L-Prolin konvertiert, und weiter durch das Enzym Prolyl-4-Hydroxylase (P4H) zu Hydroxyprolin hydroxyliert. Hydroxyprolin ist entscheidend für die Stabilität des Triple-Helix-Moleküls in den Kollagenfasern [9]. Arginase konvertiert nicht nur L-arginin zu L-Ornithin und Harnstoff, sondern spielt auch eine wichtige Rolle bei der Geweberegeneration, Zellproliferation und zeigt anti-entzündliche Eigenschaften [34, 45].

Unsere Messungen zeigten, dass die Konzentration von Hydroxyprolin in den Keratozyten von KC-Patienten ebenfalls erniedrigt ist, was eine Erklärung für die verminderte Stabilität der Hornhaut sein könnte. Um diesen Mechanismus weiter zu erforschen, wurden in der dritten Studie weitere Parameter aus dem L-Arginin Metabolismus analysiert.

NF-κB und iNOS Expression in Keratokonus-Keratozyten – Hinweise auf eine entzündliche Komponente? (Veröffentlichung 3)

In dieser dritten Studie wurden die beiden Pfade des L-Arginin Metabolismus untersucht. Es gibt zwei bekannte Isoenzyme der Arginase (Arg I und Arg II). Arginase I wird hauptsächlich in der Leber ausgebildet, während man Arginase II in vielen anderen Zelltypen findet [44]. Unsere Experimente konnten zeigen, dass lediglich Arginase II von den Keratozyten gebildet wird.

Das konkurrierende Enzym der Arginase ist die Stickstoffmonoxid-Synthase (NO-Synthase), von der 3 Isoformen bekannt sind. In Keratozyten wird lediglich wie von uns gezeigt, die induzierte NO-Synthase (iNOS) ausgebildet. Grundsätzlich wird diese Isoform nur durch Entzündungsreize aktiviert, es gibt jedoch einige Zellarten wie z.B. glatte Muskelzellen, Epithelzellen der Luftwege und retinale Zellen, die ebenfalls iNOS exprimieren, ohne an entzündlichen Prozessen beteiligt zu sein [15].

Während eines entzündlichen Prozesses benötigen die Zellen zur Erregerabwehr mehr Stickstoffmonoxid, welches als freies Radikal wirken kann [8]. Welche Rolle konstitutiv exprimierte iNOS hat, ist noch nicht vollständig geklärt [15]. Die Keratozyten von KC-Patienten exprimieren in der Zellkultur mehr iNOS mRNA als die gesunden Vergleichszellen, allerdings ist die daraus resultierende Proteinmenge nicht unterschiedlich. In den letzten Jahren mehren sich die Hinweise auf eine subklinische entzündliche Komponente bei der Pathogenese des Keratokonus. Eine erhöhte iNOS Expression, die wir in den Keratozyten nachweisen konnten, unterstützt diese These. Um diese Theorie zu untermauern, untersuchten wir zusätzlich den Nuklear Faktor kappa B (NF-κB) der ein Schlüssel-Gen bei entzündlichen Prozessen darstellt und ein Transkriptionsfaktor unter anderem für iNOS ist. Wie unsere Experimente zeigen, ist die NF-κB mRNA Expression und die Protein Expression in KC-Keratozyten im Vergleich zu den Kontrollzellen erhöht. Das könnte die erhöhte iNOS mRNA Expression der KC-Keratozyten erklären.

Um den regulativen Mechanismus der iNOS näher zu beleuchten, wurde die iNOS mit einem spezifischen Inhibitor (1400W) blockiert, um den Einfluss der iNOS auf die zelluläre Produktion von Urea und NO zu untersuchen.

Nach der Arbeitshypothese sollte durch die Hemmung der iNOS weniger NO produziert werden, welches als Nitrit messbar ist, und gleichzeitig die Arginase mehr L-Arginin zur Verfügung haben, um mehr Harnstoff und L-Ornithin zu bilden, und im weiteren Verlauf einen möglichen Einfluss auf das Hydroxyprolin zeigen.

Die Ergebnisse zeigten allerdings, dass der Inhibitor 1400W nur bei den KC-Keratozyten Einfluss auf die Konzentration von Nitrit hatte, das vermindert messbar war, allerdings bei gleichzeitiger Reduzierung des Harnstoffs. Der Inhibitor zeigte keinen Effekt auf Nitrit und Harnstoff Konzentrationen bei normalen Keratozyten.

Diese Ergebnisse und die erhöhte NF-κB Expression sprechen für eine inflammatorische Komponente beim Keratokonus, deren Ursachen in weiteren Experimenten untersucht werden sollte, um mehr Einblick in die Pathogenese der Erkrankung zu erhalten.

Das Wissen über die pathologischen metabolischen Veränderungen mit einer pathogenetischen Ursache in Verbindung zu bringen ist von enormer Bedeutung hinsichtlich der möglichen Behandlungsmethoden für die Patienten. Nur durch ein besseres Verständnis der Erkrankung ist es möglich, neue, weniger invasive Behandlungsmethoden für die Patienten zu entwickeln und die Progression der Erkrankung zu verlangsamen.

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Urea, Uric Acid, Prolactin and fT4 Concentrations in Aqueous Humor of Keratoconus Patients

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ABSTRACT

Purpose: Keratoconus is a noninflammatory disease of the cornea associated with progressive thinning and conical shape. Metabolic alterations in the urea cycle, with changes in collagen fibril stability, oxidative stress, thyroid hormones and prolactin with regulatory effect on biosynthesis and biomechanical stability of corneal stroma, may all play a role in keratoconus etiology. Our purpose was to determine urea, uric acid, prolactin and free thyroxin (fT4) concentrations in human aqueous humor (hAH) of keratoconus and cataract patients.

Methods: hAH was collected from 100 keratoconus (penetrating keratoplasty) (41.9 ± 14.9 years, 69 males) and 100 cataract patients (cataract surgery) (71.2 ± 12.4 years, 58 males). Urea, uric acid, prolactin and fT4 concentrations were measured by Siemens clinical chemistry or immunoassay system. For statistical analysis, a generalized linear model (GLM) was used.

Results: Urea concentration was 11.88 ± 3.03 mg/dl in keratoconus and 16.44 ± 6.40 mg/dl in cataract patients, uric acid 2.04 ± 0.59 mg/dl in keratoconus and 2.18 ± 0.73 mg/dl in cataract groups. Prolactin concentration was 3.18 ± 0.34 ng/ml in keratoconus and 3.33 ± 0.32 ng/ml in cataract patients, fT4 20.57 ± 4.76 pmol/l in KC and 19.06 ± 3.86 pmol/l in cataract group. Urea concentration was effected through gender ($p = 0.039$), age ($p = 0.001$) and diagnosis ($p = 0.025$). Uric acid concentration was not effected through any of the analyzed parameters ($p > 0.056$). Prolactin and fT4 concentration were effected only through diagnosis ($p = 0.009$ and $p = 0.006$).

Conclusions: Urea and prolactin concentrations are decreased, fT4 concentration is increased in aqueous humor of keratoconus patients, and uric acid concentration remains unchanged. Urea concentration in aqueous humor is also increased in older and male patients. Therefore, metabolic disorder and hormonal balance may both have an impact on keratoconus development. Further studies are necessary to assess the specific impact.

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Aqueous humor; fT4; keratoconus; prolactin; urea; uric acid

Introduction

Keratoconus (KC) is a corneal disease characterized by progressive thinning and conical shape. The etiology of keratoconus is unknown, but involvement of genetical and environmental components is widely accepted.[1,2]

Human aqueous humor (hAH) is known to regulate many functions of the eye. hAH provides nutrition, growth factors and electrolytes. It also removes excretory products from metabolism and stabilizes the ocular structure.[3] Several reports have shown differences in the composition of hAH or tear fluid in keratoconus patients compared to healthy subjects.[4–6] Jäger et al. demonstrated significant lower urea concentrations in tear film of patients with dry-eye syndrome (DES), which is often connected to keratoconus and compared to healthy controls.[7] Urea is the end product of nitrogen metabolism and is produced in the urea cycle. Arginase catalyzes the conversion of arginine to urea and ornithine. Arginine is indirectly involved in collagen synthesis, by the support of nitric oxide (NO), which is synthesized from arginine. The synthesis of nonessential amino acids may play an important role in keratoconus development.

Uric acid is a small molecule and acts as an antioxidant. It is part of the radical scavenging system and is able to protect cells from damage, caused by oxidative stress. Uric acid and ascorbic acid provide the main part of total antioxidant activity in tear fluid.[8] Less antioxidant levels may lead to insufficient elimination of reactive oxygen species (ROS) and therefore promote cell damage.

Prolactin-inducible protein (PIP) is a secretory glycoprotein, which was found in the proteome of tear fluid.[9] PIP is up-regulated by prolactin and androgens and down-regulated by estrogens. Priyadarsini et al. demonstrated a decreased PIP mRNA expression in tear fluid of keratoconus patients compared to normal controls.[10] Prolactin is produced not only by the anterior pituitary gland, but also by human skin and shows non-mammatropic effects.[11] Furthermore, prolactin exhibits an effect on collagen organization in 3D tissue culture models.[12]

Thyroid hormones are known to have effects on collagen biosynthesis in many cell types.[13] It was shown that the

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corneal thinning in progressive keratoconus is accompanied by changes in distribution and orientation of collagen within the corneal stroma.[14] Cases of acute keratoconus induced by hypothyroxinemia during pregnancy are also described.[15]

Our purpose was to determine urea, uric acid, prolactin and free thyroxin (fT4) concentrations in human aqueous humor (hAH) of keratoconus and cataract patients.

Methods

Ethical considerations

The study was approved by the Ethics Committee of Saarland and Rheinland-Pfalz (no 56/14), and informed consent was obtained from all participants. Only patients with an age older than 18 years were included in the study.

Subjects

Demographic characteristics of the patients are displayed in Table 1.

HAH was collected during elective penetrating keratoplasties, following paracentesis, from 100 KC patients (Pentacam stages 2-4). Penetrating keratoplasty was performed in keratoconus patients, if best corrected visual acuity (corrected with contact lenses or glasses) was not above 0.4.

100 hAH samples were also collected during cataract surgery, following paracentesis, before any further manipulation.

One percent of KC patients was previously operated with cataract, and 10% of keratoconus patients had a cataract (beginning stage of cataract, without planning cataract surgery simultaneously with penetrating keratoplasty) in the analyzed subjects. These data were respected for the statistical analysis. Neither keratoconus nor cataract patients undergo other previous ocular surgeries.

Three percent of the cataract group patients had Fuchs' corneal dystrophy, and 1% had glaucoma in both groups. No other ocular diseases were known in the analyzed patients of both groups.

There was no patient with diabetes in the KC group, but 3% of cataract patients were diagnosed and treated with diabetes mellitus. Ten percent of keratoconus patients had thyroid gland dysfunction, which was 13% in the cataract group.

Sample volumes of 50-100 µl were collected for both groups and frozen at -80°C until measurements.

Measurement

Blood urea nitrogen (BUN, for simplification, we use throughout the text urea) and uric acid concentrations were assessed by the ADVIA 1800 Chemistry System (Siemens Healthcare Diagnostics GmbH, Erlangen, Germany).

Table 1. Demographic characteristics of keratoconus and cataract patients.

	Keratoconus	Mean age ± SD (years)	Cataract	Mean age ± SD (years)
Total	100	41.9 ± 14.9	100	71.2 ± 12.4
Female	31	48.8 ± 15.2	42	73.8 ± 10.7
Male	69	38.9 ± 13.8	58	69.4 ± 13.2

To measure urea concentration, the method of Roch-Ramel was used determining the urease-catalyzed release of ammonia by glutamate dehydrogenase enzyme. This enzymatic method was developed to estimate urea concentrations in nanoliter volume specimens.

Uric acid concentration was determined using the reaction of Fossati. In this assay, uric acid is oxidized to allantoin and hydrogen peroxide. The peroxides change to a red colored reaction product, which can be measured at a wavelength of 505 nm.

Prolactin and fT4 concentrations were assessed by the ADVIA Centaur XPT Immunoassay System (Siemens Healthcare Diagnostics GmbH, Erlangen, Germany). FT4 concentration was determined by a sandwich chemiluminescent immunoassay and prolactin concentration by a competitive chemiluminescent immunoassay.

The instruments were calibrated, and internal quality control was performed using the same lot of manufacturer's control and calibration material.

Statistical analysis

The statistical analysis was performed using SPSS software (version 19.0). In this study, the "generalized linear model" (GLM) was used, which also analyzed the effect of gender, age and diagnosis on urea, uric acid, fT4 and prolactin concentrations in aqueous humor of the patients. GLMs are more flexible and better suited for the analysis of relationships, which can be poorly represented by classical Gaussian distributions. P values < 0.05 were considered statistically significant.

Results

Urea, uric acid, prolactin and fT4 concentrations in aqueous humor of keratoconus and cataract patients are shown at Figures 1–4.

Urea concentration was 11.88 ± 3.03 mg/dl in keratoconus and 16.44 ± 6.40 mg/dl in cataract patients, uric acid 2.04 ± 0.59

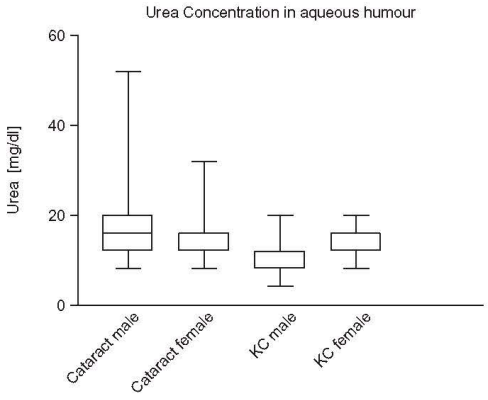

Figure 1. Urea concentration of male and female patients with diagnosis of keratoconus or cataract.

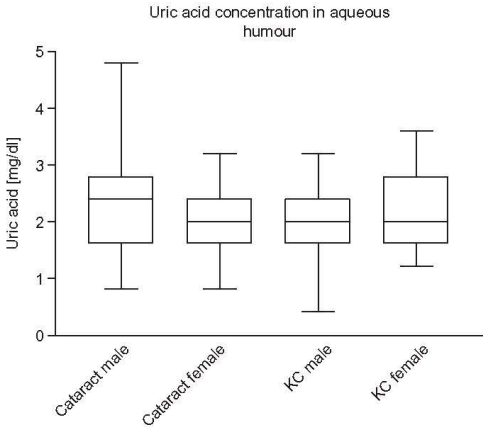

Figure 2. Uric acid concentration of male and female patients with diagnosis of keratoconus or cataract.

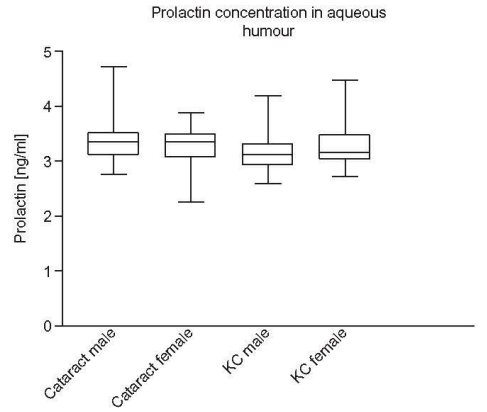

Figure 3. Prolactin concentration of male and female patients with diagnosis of keratoconus or cataract.

mg/dl in keratoconus and 2.18 ± 0.73 mg/dl in cataract groups. Prolactin concentration was 3.18 ± 0.34 ng/ml in keratoconus and 3.33 ± 0.32 ng/ml in cataract patients, fT4 20.57 ± 4.76 pmol/l in KC and 19.06 ± 3.86 pmol/l in cataract group.

The effect of age, gender and diagnosis on urea, uric acid, fT4 and prolactin concentrations in aqueous humor, using a generalized linear model (GLM), is displayed in Table 2.

Urea concentration was decreased in patient with keratoconus and effected through gender ($p = 0.039$), age ($p = 0.001$) and diagnosis ($p = 0.025$). Uric acid concentration was not effected through any of the analyzed parameters ($p > 0.056$). Prolactin ($p = 0.009$) and fT4 ($p = 0.006$) concentrations were

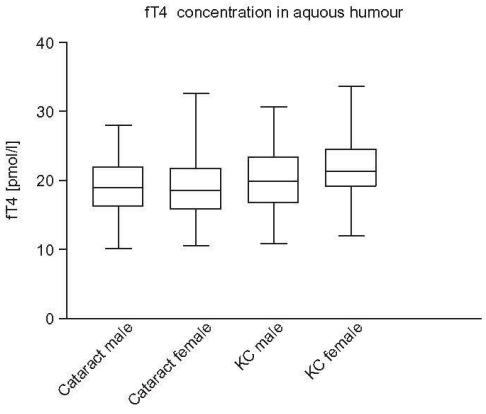

Figure 4. fT4 concentration of male and female patients with diagnosis of keratoconus or cataract.

Table 2. Effect of age, gender and diagnosis on urea, uric acid, prolactin and fT4 concentrations in aqueous humor.

	Urea significance	Uric acid significance	Prolactin significance	fT4 significance
Gender	**0.039**	0.056	0.811	0.533
Age	**0.001**	0.155	0.578	0.184
Diagnosis	**0.025**	0.909	**0.009**	**0.006**

decreased in keratoconus group. Prolactin and fT4 were effected through diagnosis but were unaffected by gender and age ($p > 0.18$).

Discussion

Keratoconus is known to be a disease of multifactorial origin. A major characteristic of KC is corneal thinning, which is associated with destruction of the extracellular matrix. Several genes encoding collagen were associated with KC.[16]

However, metabolic disorders, like changes in the urea cycle, might also influence the development of keratoconus.[4] The conversion of arginine to urea and ornithine is the last step in the urea cycle. Ornithine is converted to proline, which is hydroxylized to hydroxyproline. Hydroxyproline is the main component of collagen and is responsible for its stabilization. Our study, which included 100 keratoconus patients, revealed lower urea concentration in hAH of KC patients, than in control cataract patients, independent from gender and age. This supports the previous suspicion that keratoconus is connected with an impairment of the urea cycle.

Oxidative stress is also discussed as a potential factor in keratoconus development. Keratoconus corneas are known to have a decreased total antioxidant capacity.[17] Uric acid, as a nonenzymatic antioxidant, should protect keratocytes from cell damage by reactive oxygen species (ROS). Uric acid has a high reactivity with singlet oxygen. It is present in serum,

tear fluid and aqueous humor, and increased uric acid concentrations were observed in tear film of keratoconus patients.[5,18] In hAH, we could not detect an effect of the diagnosis of keratoconus on uric acid concentrations. It might be possible that other nonenzymatic antioxidants like ascorbic acid, glutathione, L-tyrosine, L-cysteine or ferritin also show different distribution in tear film and aqueous humor of these patients, compensating the decreased antioxidant capacity in KC.

Prolactin is connected with cell proliferation and was found in lacrimal glands. It is known as an inducer of prolactin-inducible protein, which is involved in the maintenance control of metabolic function. Prolactin is further a neuroendocrine regulator of keratin transcription and protein production. Keratins are proteins that form the flexible cytoskeleton scaffold in the cytoplasm of epithelial cells and therefore are responsible for the structural stability of the cells. Keratin K3, K4, K5, K12, K14, K 16 and K24 were found in corneal epithelium, and K5 and K12 were identified as corneal stromal proteins. Keratin K14 and K16 showed an up-regulation in keratoconus epithelium.[19] Prolactin up-regulates human keratin K5, K14, K15 and K19 *is situ*.[20] The fact that prolactin concentration is decreased in aqueous humor of keratoconus patients might be important evidence, how metabolic disorders may have an impact on keratoconus disease. Changes in prolactin concentration of hAH could lead to changes in keratin expression, either in corneal epithelial cells or in corneal stromal cells. It should be investigated, whether prolactin influences keratin production of epithelial and stromal cells of the cornea and how this results in changes of corneal structural stability.

Thyroxin increases the basal metabolic rate in human cells. FT4 concentration in aqueous humor of keratoconus patients is increased, independent from gender and age of the patients. Beradi et al. showed in their study an increase in collagen production of tenocytes after thyroid hormone treatment.[12] However, the effect of increased fT4 levels in aqueous humor of keratoconus patients on keratocytes or collagen synthesis of keratocytes is not described, yet.

Interestingly, McKay et.al. measured altered hormone levels in saliva samples of KC patients, compared to healthy controls. Therefore, they also suggested that hormonal regulation plays a role in keratoconus pathology. In addition, they found uric acid and urea concentration pattern in saliva of KC patients similar to our present results in aqueous humor of KC patients.[21] This also supports our hypothesis that metabolic disorders may influence the development of keratoconus. Therefore, many questions still remain to be clarified in order to explain the metabolic and hormonal changes in aqueous humor of keratoconus patients.

In summary, urea and prolactin concentrations are decreased and fT4 concentration is increased in aqueous humor of keratoconus patients, whereas uric acid concentration remains unchanged. Overall, urea concentration in aqueous humor is also increased in older and male patients. Therefore, metabolic disorder and hormonal balance may both have an impact on keratoconus development.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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BASIC SCIENCE

Arginase activity, urea, and hydroxyproline concentration are reduced in *keratoconus keratocytes*

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Abstract
Purpose Keratoconus (KC) is a disease characterized by thinning and deformation of the cornea, but its etiology remains unknown. Seventy percent of the corneal stroma consists of collagen, which is composed of three intertwined polypeptide chains with *glycine-hydroxyproline-proline* repeats along their sequence. *Arginase* is a cytoplasmatic enzyme and catalyzes the conversion of arginine to urea and ornithine, which serves as a precursor for the endogenous synthesis of proline and hydroxyproline. The purpose of this study was to analyze arginase activity, as well as collagen and urea formation in *normal* and *KC-keratocytes* and to determine the impact of urea on keratocyte viability and proliferation in vitro.
Methods Primary human keratocytes were isolated by digestion in collagenase (1.0 mg/mL) from surgically removed corneas of eight keratoconus patients and eight normal human corneal buttons and cultured in DMEM/Ham's F12 medium supplemented with 5 % fetal calf serum. Arginase activity and urea concentration were measured in cell-lysates, hydroxyproline concentration in supernatant of cultured keratocytes using colorimetric assay. Cell viability and cell proliferation of cultured keratocytes were assessed after treatment with urea at concentrations up to10 mM for 24 h using assays for metabolic activity and DNA replication.
Results Arginase activity and urea concentration in KC-keratocytes decreased by about 50 % compared to normal keratocytes ($p = 0.003$ and $p = 0.008$). Hydroxyproline synthesized by cultured KC-keratocytes was also approximately 50 % less compared to normal keratocytes ($p = 0.02$) and this difference decreased following treatment with 5.0 or 10.0 mM urea ($p = 0.02$; 0.03), without any change in cell viability ($p > 0.09$). However, the urea treatment increased modestly (by 20 %) the proliferation rate of KC-keratocytes ($p = 0.04$; 0.04; 0.04), without any effect on normal cultured keratocytes ($p > 0.09$).
Conclusions We identified suppressed arginase activity in the metabolic program of cultured *keratoconus keratocytes*. The level of urea, as one product of the enzyme arginase was also decreased. This results in impaired collagen synthesis, evidenced in the culture by reduced hydroxyproline concentration. In addition, our data showed that the other product of the arginase reaction, urea supports the proliferation of KC-keratocytes, without changes in their viability. The metabolic reprogramming of *keratoconus keratocytes* and its impact on development of a clinically detectable keratoconus disease has to be further analyzed.

Keywords Urea · Keratoconus · Keratocytes · Arginase · Hydroxyproline concentration · Viability · Proliferation

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Introduction

Keratoconus (KC) is a disease characterized by thinning and deformation of the cornea. Patients develop first symptoms usually in their early teens to early 20s with

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an incidence of about 1/2000 [1]. Keratoconus starts usually during puberty with progression until the third decade of life [2]. The etiology of keratoconus remains unknown, but some researchers suggest involvement of inflammatory enzymes beside genetical components [3–5]. Different proteases like matrix-metalloproteinase-1 (MMP-1) [6], MMP-9, and MMP-13 [7] may be involved in keratoconus disease.

Thinning of a keratoconus cornea occurs with degradation of the extracellular matrix. Seventy percent of the corneal stroma consists of collagen, predominantly of type I collagen (80–90 %) and of collagen V [8, 9]. The fibril-forming collagens are composed of triple helical subunits, the stability of which is based on the repeating glycine-X-Y amino acid triplets in the structure of the intertwined polypeptide chains (38 % of position X in these repeats is occupied by *hydroxyproline* and 28 % of position Y by *proline*) [10]. Hydroxyproline is formed by a posttranslational hydroxylation of proline in protocollagen and thus its quantity depends on the rate of collagen synthesis.

Arginase is an essential enzyme in tissue regeneration, cell proliferation, and anti-inflammation [11–13]. Arginase, as a cytoplasmic enzyme catalyzes the conversion of arginine to *urea* and ornithine. Ornithine will be further converted by ornithine aminotransferase and pyrroline-5-carboxylate reductase to proline, which is incorporated in protocollagen, the precursor of collagen and some of these proline residues are further transformed to hydroxyproline (Fig. 1). Therefore, arginase may play an essential role in collagen production of the cells, also in keratoconus patients.

Interestingly, in a previous study, we detected decreased *urea* concentration in aqueous humour of keratoconus patients compared to controls [14]. In addition, Jäger et al. have shown that urea concentration in tear film of dry eye subjects is decreased compared to healthy controls [15]. Keratoconus is often associated with dry-eye syndrome (DES), which results in symptoms of discomfort, tear film instability caused by increased osmolarity of the tear film [16].

These findings raise the hypothesis that changes related to urea metabolism may be part of the keratoconus disease phenotype. In view of the known disturbances of collagen turnover in keratoconus patients, the current study was undertaken in attempt to address the possibility that impaired arginase-dependent supply of proline could result in impaired collagen synthesis and thus contribute to the pathomechanism of keratoconus disease.

Therefore, in the current study, our purpose was to analyze arginase activity, as well as urea concentration and hydroxyproline concentration of *normal* and *keratoconus keratocytes* and to define the impact of urea on keratocyte viability and proliferation.

Materials and methods

Ethical considerations

The study was approved by the ethics committee of Saarland (Number 263/15), informed consent was obtained from all patients with keratoconus, before keratoplasty.

Materials

Dulbecco's Modified Eagle Medium: (Nutrient Mixture F-12; DMEM/F12); fetal calf serum (5 %); P/S (1 % of 10,000 u penicillin/10 mg streptomycin per mL); 0.05 % trypsin/0.02 % ethylenediaminetetra-acetic acid (EDTA), PBS, CelLytic™, Urea assay kit (Product No MAK006), and Arginase activity assay kit (Product No MAK112) were purchased from Sigma-Aldrich® GmbH (Deisenheim, Germany), alamarBlue® from Invitrogen (Product No DAL1025, Karlsruhe, Germany). Collagenase A, Dispase II, and BrdU Proliferation-ELISA (Product No 11647229001) were obtained from Roche Diagnostics (Mannheim, Germany). Colorimetric assay for Hydroxyproline was purchased from Chondrex. Inc. (Product No 6017, Redmond, WA, USA.

Isolation of primary human corneal keratocytes

Eight human normal corneas were obtained from the LIONS Cornea Bank Saar-Lor-Lux, Trier/Westpfalz and eight corneas from keratoconus patients from elective penetrating keratoplasties. Unfortunately, data on vascular disease or diabetes of the analyzed patients (keratoconus patients and controls) were not collected for the present study. To isolate keratocytes, the human corneoscleral buttons were aseptically rinsed in phosphate-buffered saline (PBS) before removal of the endothelium including Descemet's membrane by sterile surgical disposable scalpel. A central corneal button with epithelium was cut using a 8.0 mm hand-held trephine and thereafter incubated in culture medium containing 2.4 U/mL Dispase II for 4 h at 37 °C. Thereafter, 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 h at 37 °C. The digested tissue and cells were pipetted three times and centrifuged at 800 g for 7 min and finally resuspended in 1.0 mL culture medium, which consisted of basic medium (DMEM/F12) supplemented with 5 % FCS and 1 % P/S.

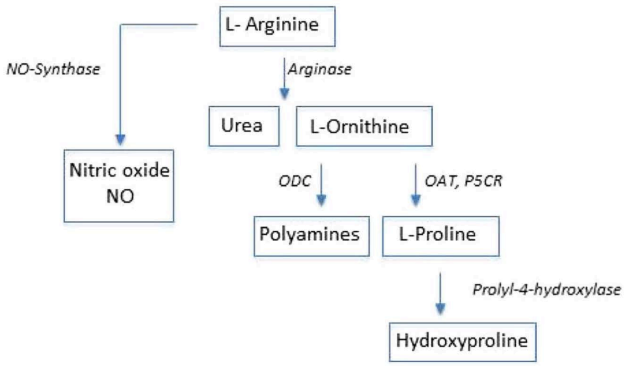

Fig. 1 *Arginase*, as a cytoplasmic enzyme catalyzes the conversion of L-arginine to L-ornithine and urea. Ornithine will be further transformed by the sequential action of *ornithine aminotransferase* (*OAT*) and *pyrroline-5-carboxylate reductase (P5CR)* to proline. Following the translation of protocollagen, some of its proline residues are hydroxylated to hydroxyproline by *prolyl-4-hydroxylase*. Ornithine is also converted by the *enzyme ornithine decarboxylase* (*ODC*) to polyamines. The competing enzyme for the substrate L-arginine is nitric oxide synthase (NO-Synthase), which catalyzes the reaction to nitric oxide

The cell suspension was seeded in 6-well plates and the medium was changed 24 h 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/0.02 % ethylenediaminetetra-acetic acid (EDTA) for 3 to 5 min, and the passage four to eight of cells was used for experiments. Normal keratocyte cell cultures were used as control cells.

Arginase activity measurement

After the cultured cells reached confluence, lysate of 3×10^6 normal ($n = 8$) or 3×10^6 KC keratocytes ($n = 8$) was prepared using 10 mM Tris/HCL buffer (pH 7.4) containing 0.4 % (w/v) Triton™ X-100 with three additional freeze and thaw cycles. Following centrifugation, the supernatant was used for the assay. The test was performed according to the manufacturer's protocol. Shortly, arginase from samples catalyzes the conversion of arginin to urea and ornithine. The produced urea specifically reacts with the substrate to generate a colored product, proportional to the arginase activity. The absorbance was measured at 550 nm using an absorbance microplate reader (TECAN infinite 50, TECAN Deutschland GmbH, Crailsheim, Germany). The arginase activity of each sample was calculated using the absorbance of a standard solution.

Protein measurement

After taking the supernatant for hydroxyproline determination, the total protein concentration of each well was measured to calculate the amount of hydroxyproline per mg protein, as follows. Following detachment of the cells with 150 μL CelLytic™, protein quantity was determined according to the method of Bradford, which is based on the formation of a complex between the dye, Brilliant blue G, and proteins in solution. The absorbance was measured at 595 nm and the concentrations were quantified using bovine serum albumin (BSA) as standard.

Urea concentration measurement in keratocytes

Lysate of 5×10^6 normal ($n = 5$) and KC keratocytes (KC) ($n = 5$) was prepared using 10 mM Tris/HCL buffer (pH 7.4) containing 0.4 % (w/v) Triton™ X-100 with three additional freeze and thaw cycles. After centrifugation, the supernatant was used for the assay. The test was performed according to the manufacturer's protocol: Urea concentration was determined by a coupled enzyme reaction, which results in a colorimetric product, proportional to the urea concentration. The optical density was measured at 550 nm using an absorbance microplate reader (TECAN infinite 50). The urea concentration of each sample was calculated using the optical density of a standard solution.

Urea treatment of keratocyte cell cultures before hydroxyproline measurement

Keratocytes were seeded in a 24-multiwell plate at a density of 8×10^3 cells/cm^2 in culture medium of 1 mL per well. After a growth period of 48 h the culture medium was changed to culture medium containing urea concentrations of 0, 5.0, and 10.0 mM for 24 h at 37 °C for 24 h.

The supernatant was stored at −80 °C until hydroxyproline measurement.

Hydroxyproline measurement

Hydroxyproline measurement in the supernatant of urea treated keratocytes was performed by a colorimetric assay kit with linear detection ranges of 6–400 µg/ml. Measured concentrations below the lower threshold of this range were considered zero. The measurements were performed following the manufacturer's protocol. First, supernatant of keratocytes was hydrolyzed with 10 N HCL at 120 °C for 24 h. In order to avoid evaporation of fluid resulting in falsely high results, the authors used an incubator and special glass vials with a corresponding lid, recommended for temperatures up to 150 °C (Rotilabo glass vials, KE27.1, Screw-on lid, NL96.1, Carl Roth, Karlsruhe, Germany)."

Then, sample solutions were transferred to microcentrifuge tubes and were spun at 10,000 rpm for 3 min. Thereafter, the supernatant of the solution was pipetted into a 96-well plate and was treated with chloramine-T and Ehrlich reagent (dimethylaminobenzaldehyde). Additional microplate wells were filled with known dilutions of hydroxyproline and were used to create a calibration curve. The optical density of each well was measured with an absorbance microplate reader at 550 nm (TECAN infinite 50). Hydroxyproline content in samples was then determined using regression analysis. In each well, the concentration of hydroxyproline was normalized to the cell protein concentration of the respective well.

Keratocyte viability using different urea concentrations

Cell viability was evaluated using the alamarBue® assay as follows: human keratocytes were seeded in 24-well cell culture plates at a density of 7.5×10^3 cells/cm^2. After a growth period of 24 h, the culture medium was changed to culture medium containing urea concentrations of 0, 0.5, 1.0, 5.0, and 10.0 mM at 37 °C for 24 h. Subsequently alamarBlue® solution was diluted with culture medium for a final concentration of 10 % and 500 µL of this solution was added to each well. As a negative control, alamarBlue® solution was added to a well without cells. Absorbance was measured at 550 nm using a 96-well microplate reader (Tecan infinite F50).

Keratocyte proliferation using different urea concentrations

To detect the impact of urea on the proliferation rate of the keratocytes, the proliferation was determined with the proliferation ELISA-BrdU kit by the measurement of BrdU(5-bromo-2'deoxyuridine) incorporation in the newly synthesized cellular DNA. Keratocytes were seeded in a 96-multiwell plate at a density of 6×10^3 cells/cm^2 in culture medium of 100 µL per well. After a growth period of 24 h, the culture medium was changed to culture medium containing urea at 0, 0.5, 1.0, 5.0, and 10.0 mM at 37 °C for 24 h. The test was performed according to the manufacturer's protocol. Briefly, BrdU was added to the keratocytes and incubated at 37 °C for 4 h. After removing the culture medium, the cells were fixed with FixDenat, provided with the test kit, followed by the incubation with anti-BrdU-POD (peroxidase), which binds the incorporated DNA. After adding the substrate solution, the immune complex was detected using an absorbance microplate reader (Tecan Infinite 50).

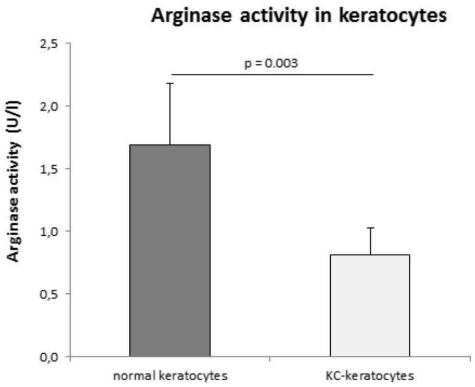

Fig. 2 Arginase activity in normal and keratoconus (KC) keratocytes ($n = 8$). Significant difference is indicated

Statistical Analysis

For statistical analysis the GraphPad Prism 2.01 was used. Data are represented as mean ± standard deviation (SD).

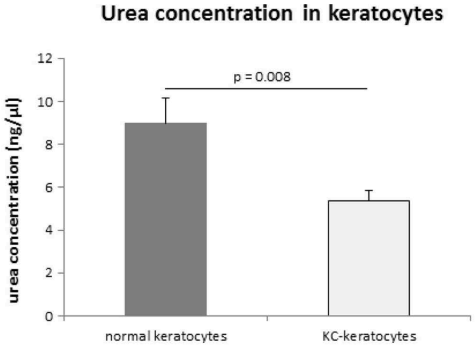

Fig. 3 Urea concentration in normal and keratoconus (KC) keratocytes ($n = 5$). Significant difference is indicated

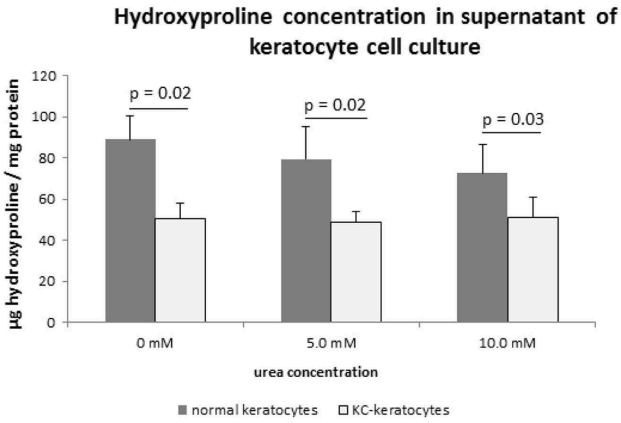

Fig. 4 Hydroxyproline concentration in cultures of normal and keratoconus (KC) keratocytes 24 h after urea treatment ($n = 6$). Hydroxyproline concentration of keratocytes was significantly less in keratoconus cultures compared to normal keratocytes adding 0, 5.0, or 10.0 mM concentrations of urea to the cultures ($p = 0.02$, 0.,02 and 0.03)

Statistical analysis was performed using Wilcoxon-Mann–Whitney Test. p values below 0.05 were considered statistically significant.

Results

Arginase activity in KC-keratocytes was significantly decreased compared to normal keratocytes ($p = 0.003$), as shown in Fig. 2. In line with this finding, the concentration of the arginase reaction product, urea, was also significantly lower in cultured KC-keratocytes (5.36 ng/µL) than in normal keratocytes (8.89 ng/µL; $p = 0.008$) ($p = 0.008$) (Fig. 3). The significantly lower hydroxyproline concentration in the KC-keratocyte cultures indicated impaired collagen synthesis compared to normal keratocytes (Fig. 4), although treatment with 5.0 or 10.0 mM urea did not change significantly the viability of KC-keratocytes compared to controls ($p > 0.09$) (data not shown). The difference in the hydroxyproline content of normal and KC-keratocytes decreased after 24-h urea exposure, but remained significant ($p = 0.02$ and 0.03) (Fig. 4). The treatment of normal keratocytes with different urea concentrations did not change cell proliferation significantly ($p > 0.09$). However, proliferation of KC-keratocytes was significantly ($p < 0.05$) increased by exposure to 0.5, 1.0, or 10.0 mM urea, compared to *keratoconus keratocyte* cultures without urea treatment (Fig. 5).

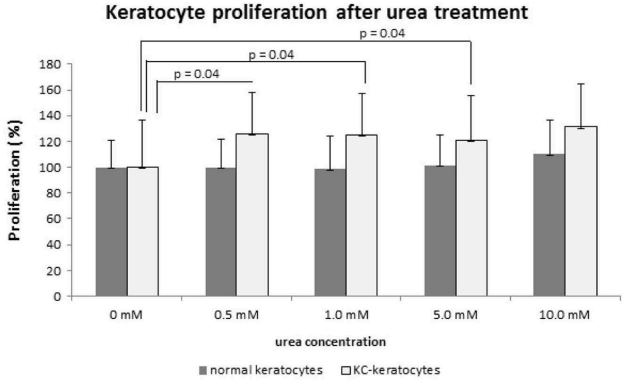

Fig. 5 Proliferation rate of normal and keratoconus (KC) keratocytes ($n = 6$). Incorporation of BrdU(5-bromo-2'deoxyuridine) in the newly synthesized cellular DNA was measured after 24-h exposure to urea at the indicated concentrations in the culture medium. The absorbance of untreated keratocytes was set to 100 %. Significant differences are indicated. The treatment of normal keratocytes with different urea concentrations did not change keratocyte proliferation significantly compared to control cells ($p > 0.09$). However, proliferation of KC keratocytes was significantly increased using 0.5, 1.0, or 10.0 mM concentrations of urea ($p = 0.04$; 0.04; 0.04), compared to KC keratocyte cultures without urea treatment

Discussion

Keratoconus is a severe disease of the cornea, resulting in thinning and conical shape [1]. There is an association between keratoconus and collagen disorders like Ehlers-Danlos syndrome and mitral valve prolapse [2]. However, the exact causes of keratoconus remain unknown.

In patients with keratoconus, the tissue develops biomechanical instability, which results in conical shape. The production of collagen by corneal keratocytes is a major factor in biomechanical stability and shape of the cornea [17]. Impairment of collagen synthesis may be a possible reason for the development of keratoconus.

Recently, the literature on the role of arginase in the immune system has increased enormously, as it has a crucial role in various aspects of inflammation such as inflammation-triggered immune dysfunction, tumor immune escape, immunosuppression, and immunopathology of infectious disease [18]. Arginase is a manganese metalloenzyme, which plays a major role in collagen synthesis. Arginase is responsible for the conversion of L-arginine to L-ornithine and urea. L-ornithine is further converted to proline. Proline is partially hydroxylated into hydroxyproline that stabilizes the triple helical subunits of collagen [19]. Adequate amount and activity of the enzyme arginase is essential for proline supply in keratocytes for collagen synthesis (Fig. 1).

In our current study, we found impaired arginase activity in human KC keratocytes, compared to normal keratocytes. The most conspicuous finding of our study is that arginase activity, urea concentration, and hydroxyproline concentration are reduced in *keratoconus keratocytes*. Decreased arginase activity could be proven through two independent methods in our study. First, we directly measured decreased arginase activity in KC keratocytes compared to normal keratocytes. Second, as an indirect measurement, the level of urea, as one product of the enzyme arginase was also decreased. Decreased urea concentration in aqueous humour of KC patients could also be verified in our previous work, compared to controls [14]. These previous in vivo measurement data further support results of our present in vitro experiments.

With decreased arginase activity in KC keratocytes we have also found decreased hydroxyproline concentration in KC keratocytes, compared to normal controls. It is of note that the hydroxyproline concentration in keratocyte cultures is decreased, despite the supplemented L-arginine and L-proline in the culture medium. This supports the conclusion that the exogenous proline is insufficient for cells with active collagen synthesis that require excessive amounts of this amino acid. The arginase activity of keratocytes may be the limiting step in the synthesis of collagen.

All of the above data (decreased arginase activity, urea concentration, and hydroxyproline concentration) suggest an arginase-dependent impairment of collagen synthesis in *keratoconus keratocytes*. Whether arginase activity is impaired or the level of arginase expression is reduced through inherited or acquired factors remains to be further clarified.

Nevertheless, with the use of cornea bank corneas as control group, the authors did not consider bias through post mortem induced transient tissue hypoxia interfering with NO pathway [20] which may also influence arginase activity of the cells. However, these changes may diminish following 4–6 passages of the cells. This issue should be addressed in a future study.

Another novel aspect of our work was the effect of urea on the viability, proliferation, and hydroxyproline concentration of normal and KC keratocytes. We found that urea treatment stimulated the proliferation of KC-keratocytes, but not of normal keratocytes, without any changes in the viability of either cell type. Interestingly, Moeslinger et al. also found a proliferation promoting effect of urea on macrophages [21].

Keratocytes show a downregulated *MYC*-gene (MYC, proto-oncogene protein), which is involved in cell cycle regulation [22]. MYC is a transcription factor that can bind the DNA and activate several regulation processes. The activation of MYC can promote cell growth and proliferation [23]. Urea may be involved in cell signaling to activate the *MYC*-gene for cell proliferation [22] or increasing urea concentrations slow down arginase activity and through the accumulation of arginine enhance cell proliferation [13]. These pathways have to be further analyzed in keratoconus patients.

A further reason for the decreased amount of hydroxyproline may be the reduced concentration of L-arginine through the competing nitric oxide (NO) pathway (Fig. 1). If the collagen production is impaired, NO pathway may also show altered processes. In addition, it is suggested, that the development of keratoconus is also associated with increased levels of reactive oxygen species; these may also be involved in alterations of keratoconus corneas [24]. Therefore, further experiments concerning NO production via nitric oxide synthase have to be performed using keratoconus corneas.

In summary, arginase activity, urea concentration and hydroxyproline concentration are reduced in *keratoconus keratocytes*. Urea treatment of *keratoconus keratocytes* does not change viability, but stimulates proliferation of these cells. The metabolic reprogramming of *keratoconus keratocytes* and its impact on development of a clinically detectable keratoconus disease has to be further analyzed.

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Compliance with ethical standards

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Conflict of Interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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5. Veröffentlichung 3

Erhöhte NF-κB und iNOS Expression in Keratozyten von Keratokonus-Patienten – Hinweise auf eine entzündliche Komponente?

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Klin Monatsbl Augenheilkd 2019 DOI: 10.1055/a-1002-0100

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