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Tear metabolite changes in keratoconus

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ABSTRACT

While efforts have been made over the years, the exact cause of keratoconus (KC) remains unknown. The aim of this study was to identify alterations in endogenous metabolites in the tears of KC patients compared with age-matched healthy subjects. Three groups were tested: 1) Age-matched controls with no eye disease (N = 15), 2) KC – patients wearing Rigid Gas permeable lenses (N = 16), and 3) KC – No Correction (N = 14). All samples were processed for metabolomics analysis using LC-MS/MS. We identified a total of 296 different metabolites of which >40 were significantly regulated between groups. Glycolysis and gluconeogenesis had significant changes, such as 3-phosphoglycerate and 1,3 diphosphateglycerate. As a result the citric acid cycle (TCA) was also affected with notable changes in Isocitrate, aconitate, malate, and acetylphosphate, up regulated in Group 2 and/or 3. Urea cycle was also affected, especially in Group 3 where ornithine and aspartate were up-regulated by at least 3 fold. The oxidation state was also severely affected. Groups 2 and 3 were under severe oxidative stress causing multiple metabolites to be regulated when compared to Group 1. Group 2 and 3, both showed significant down regulation in GSH-to-GSSG ratio when compared to Group 1. Another indicator of oxidative stress, the ratio of lactate – pyruvate was also affected with Groups 2 and 3 showing at least a 2-fold up regulation. Overall, our data indicate that levels of metabolites related to urea cycle, TCA cycle and oxidative stress are highly altered in KC patients.

1. Introduction

Keratoconus (KC) is a non-inflammatory corneal thinning disease (Krachmer et al., 1984). In KC, the main characteristic is the deviation from the normal corneal contour and physical structure that can have devastating effects on the patient's best-corrected visual acuity (Gordon et al., 2006; Moreira et al., 2007; Wagner et al., 2007; Wahrendorf, 2006). Changes in corneal biomechanical properties leading to corneal thinning can make the individual more susceptible to traumatic ocular injury (Al-Hussain et al., 2004). KC s main characteristic is the cone-shaped corneal protrusion and is seen in all ethnical populations, but more commonly in the Middle East. Both sexes can be affected, but males more often than females. The mean age of onset is at 17 years (Bechrakis et al., 1994). The incidence rate is still debatable, though large studies estimate 50 to 230 cases per 100,000 people in the general population (Rabinowitz, 1998). Depending on the severity, visual quality can be so adversely affected as to require surgical intervention.

The origin of the disease remains unknown and research has been extended over the last decade in order to ultimately unravel the etiology KC and may be able to correct the disease at its origin in the near future. Many genetics studies are currently on going or have been completed, but no common gene defect has been (Nielsen et al., 2013). Interestingly, less than 10% of cases are believed to be of familial origin (Aldave et al., 2006). Other onset mechanisms include mechanical eye rubbing and contact lens wearing both contributing to activation of wound-healing mechanisms and signaling pathways (Yeniad et al., 2009). Biochemical studies have also been performed indicating that the role of

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proteolytic enzyme digestion and the involvement of interleukin-1 (IL-1) are possible causative factors in some cases of KC (Rabinowitz, 1998).

It is known that the stability and quality of the tear film plays an important role in the optical quality of the eye (Montes-Mico et al., 2010). In KC there are a number of studies investigating the proteome of the tear fluids from patients diagnosed with KC compared to individuals without KC (Cheng et al., 2001; Nielsen et al., 2005, 2006; Srivastava et al., 2006). The most recent and extensive proteome study revealed (Chaerkady et al., 2013) new as well as previously reported proteins that are regulated in KC patients. The authors identified proteins from normal donor and KC corneas a total of 932 and 1157 proteins in the corneal epithelium and the stroma, respectively. Previous study on the KC epithelium detected 200–500 from which 19 were differentially expressed proteins (Srivastava et al., 2006). Another recent study identified 104 epithelial and 44 stromal proteins (Joseph et al., 2011). Between these studies there are a few proteins that are consistently been reported and regulated in KC patients. Collagen XII, transketolase, and TGFBI have all been reported to be decreased (Chaerkady et al., 2013; Cheng et al., 2001; Joseph et al., 2011) in KC stromal proteome.

The aim of this study was to identify alterations in tear metabolism of KC patients compared with age-matched healthy subjects. As Rigid Gas Permeable lenses (RGP) may pose significant mechanical stress to the cornea, tears from the KC group of patients were analyzed separately dependent on whether the patients wore RGP lenses. Corneal RGPs has been shown to induce topographical changes on the cornea in normal control individuals (Braun and Anderson Penno, 2003), (Wang et al., 2002) as well as in subjects with KC (Szczotka et al., 1996). In normal subjects, alterations in corneal curvatures were observed and were directly related to the number of years of lens wear (Braun and Anderson Penno, 2003; Wang et al., 2002). In KC subjects, alterations in corneal curvature (Hwang et al., 2010; Zadnik et al., 2005; Zadnik and Mutti, 1987), shape (Gundel et al., 1996; Zadnik et al., 2005; Zadnik and Mutti, 1987), thickness (Jinabhai et al., 2012), and anterior surface (Jinabhai et al., 2012; Zadnik and Mutti, 1987) have been reported again all linked to the long term wear of RGPs. A recent study (Romero-Jiménez et al., 2014) has shown short term corneal changes of RGPs fitted in KC subjects. Authors found that the anterior cornea flattens and increases in thickness within a 14 day period of RGP wear.

This is the first study, to the author's knowledge, to identify key metabolites in human tears for the study of KC disease in order to provide clues for the treatment of the defect. Furthermore, recent studies linking metabolomics to genomic (Adamski and Suhre, 2013; Gieger et al., 2008) profiling suggests that there may be even greater opportunities for us to approach the disease and grasp a greater understanding of the underlying mechanisms involved.

2. Methods

2.1. Subject recruitment

Thirty patients referred to the Department of Ophthalmology, Aarhus University Hospital for KC was asked to provide a tear sample for testing. All patients underwent a standard clinical examination including refraction, measurement of best corrected visual acuity, slit-lamp examination, and Pentacam HR Scheimpflug tomography. Fifteen patients referred for refractive surgery for myopia underwent similar examination and served as control group. Tears were collected in capillary glass tubes from the mid-temporal side of the tear meniscus and were collected only in the morning hours to ensure consistency. The volume obtain was on average 5–7 μl per patient. Care was taken not to stimulate tear secretion during collection.

2.2. Pentacam

All individuals participating in the study were examined using the Pentacam HR (Oculus, Optikgeräte GmbH). A variety of values were collected. As shown in Table 1 the mean age for healthy individuals was 38 (range from 31 to 47 years). For KC individuals that received RGP treatment the average age was 29.3 (range 21 to 58 years). For KC individuals with no correction the average age was 29.7 (range 20 to 51). There were no statistically significant differences in age between the study groups. The mean corneal thickness for the control group was 551.9, for the RGP treated group was 451 and for the no correction group was recorded as 455.6 (Table 1). Maximum keratometric (Kmax) average value for the controls was 43.9, the RGP group was 53.9 and the No correction group average value was 52.7 (Table 1). Patients with RGP lenses were instructed to leave lenses out for at least one week prior to tear collection.

2.3. Tear metabolite extraction

All samples were collected and processed as previously reported (Yuan et al., 2012). Briefly, samples were centrifuged (14,000 g, 10 min, 4 °C) in ice-cold 80% MeOH. Supernatants were incubated on dry ice. Plasma metabolites were extracted from the tear samples twice in 80% ice-cold MeOH. Metabolite extracts were vortexed and centrifuged (14,000 g, 10 min, 4° C). Supernatants were evaporated and stored at −80 °C until further analysis.

2.4. Targeted mass spectrometry

Targeted mass spectrometry was used for sample processing, as previously described (Karamichos et al., 2014; Webhofer et al., 2013; Yuan et al., 2012). Briefly, samples were re-suspended using 20 μL HPLC grade water and 5–7 μl was injected into a hybrid 5500 QTRAP triple quadrupole mass spectrometer (AB/SCIEX) coupled to a Prominence UFLC HPLC system (Shimadzu, Columbia, MD) (Webhofer et al., 2013). A total of 256 endogenous water soluble metabolites were analyzed using selected reaction monitoring (SRM). Some metabolites were targeted in both positive and negative ion mode, for a total of 289 SRM transitions, using positive/negative ion polarity switching. Approximately 10–14 data points were acquired per detected metabolite. Samples were delivered to the mass spectrometer via hydrophilic interaction chromatography (HILIC) where gradients were run as previously described (Karamichos et al., 2014; Webhofer et al., 2013; Yuan et al., 2012). Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v2.0 software (AB/SCIEX).

Table 1
Summary of the mean ages, corneal thickness (Ct min) and maximum keratometric (Kmax) values for Group 1: Controls, Group 2: KC-RGP, and Group 3: KC-no correction. Mean age for Group 1 was 38, Group 2 was 29.3, and for Group 3 the average age was 29.7. No statistically significant differences were found. The Ct min for Group 1 was 551, for Group 2 was 451 and Group 3 was recorded as 455. Both Groups 2 and 3 were statistically different compared to Group 1 ($p < 0.05$). Kmax average value for the controls was 43.9, the KC-RGP group was 53.9 and the KC-No correction group average value was 52.7.

	Age (years)	Ct min (um)	Kmax (D)
Control	38 ± 7.02	551.9 ± 7.96	43.9 ± 0.35
KC-RGP	29.3 ± 9.18	451 ± 6.23	53.9 ± 1.31
KC-No correction	29.7 ± 9.27	455.6 ± 12.43	52.7 ± 1.46