In vitro model suggests oxidative stress involved in keratoconus disease

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In vitro model suggests oxidative stress involved in keratoconus disease

D. Karamichos¹, A. E. K. Hutcheon², C. B. Rich⁴, V. Trinkaus-Randall⁴, J. M. Asara³ & J. D. Zieske²

¹Department of Ophthalmology, University of Oklahoma Health Sciences Center, USA, ²Schepens Eye Research Institute/Massachusetts Eye and Ear and the Department of Ophthalmology Harvard Medical School, Boston, MA, USA, ³Division of Signal Transduction/Mass Spectrometry Core, Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA, ⁴Departments of Biochemistry and Ophthalmology, Boston University School of Medicine, 80 E Concord Street, Boston, MA 02118, USA.

Keratoconus (KC) affects 1 : 2000 people and is a disorder where cornea thins and assumes a conical shape. Advanced KC requires surgery to maintain vision. The role of oxidative stress in KC remains unclear. We aimed to identify oxidative stress levels between human corneal keratocytes (HCKs), fibroblasts (HCFs) and keratoconus cells (HKCs). Cells were cultured in 2D and 3D systems. Vitamin C (VitC) and TGF-β3 (T3) were used for 4 weeks to stimulate self-assembled extracellular matrix (ECM). No T3 used as controls. Samples were analyzed using qRT-PCR and metabolomics. qRT-PCR data showed low levels of collagen I and V, as well as keratocan for HKCs, indicating differentiation to a myofibroblast phenotype. Collagen type III, a marker for fibrosis, was up regulated in HKCs. We robustly detected more than 150 metabolites of the targeted 250 by LC-MS/MS per condition and among those metabolites several were related to oxidative stress. Lactate levels, lactate/malate and lactate/pyruvate ratios were elevated in HKCs, while arginine and glutathione/oxidized glutathione ratio were reduced. Similar patterns found in both 2D and 3D. Our data shows that fibroblasts exhibit enhanced oxidative stress compared to keratocytes. Furthermore the HKC cells exhibit the greatest level suggesting they may have a myofibroblast phenotype.
Targeted metabolomics using mass spectrometry has been used to profile many diseases such as cancers from biological tissue sources such as cerebral spinal fluid (CSF), plasma and tumor tissue. In our current study, we investigated the metabolic differences between human corneal keratocytes (HCKs), fibroblasts (HCFs) and HKCs, under conventional 2D cultures and using our 3D in vitro model. Investigating both systems allow us to compare cells in a monolayer culture and cells in a self-assembled ECM, which more resembles an in vivo like condition. From these studies, our data indicated that HKCs express metabolites that are indicative of oxidative stress both in the 2D and 3D cultures.

Results

Cell morphology and real-time-PCR. We investigated the morphology of the three cell types. Both HKCs and HCFs were elongated or fibroblastic in appearance, while the HCKs were dendritic in shape. Figure 1 shows the morphological phenotype of HKCs which is very fibroblastic in appearance, while the HCKs were dendritic in shape. Both HKCs and HCFs were elongated or

<table>
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<tr>
<th>Collagen-I</th>
<th>Collagen-III</th>
<th>Collagen-V</th>
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<tr>
<td>RQ Mean</td>
<td>RQ Mean</td>
<td>RQ Mean</td>
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<tr>
<td>HCK</td>
<td>11.46 ± 0.68</td>
<td>0.07667 ± 0.0016</td>
</tr>
<tr>
<td>HCF</td>
<td>2.84 ± 0.30</td>
<td>2.27 ± 0.15</td>
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<tr>
<td>HKC</td>
<td>2.763 ± 0.29</td>
<td>8.637 ± 0.61</td>
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Predicted Pathways of Regulation. Our system allows us to investigate the levels of various metabolites, both in cells that are conventionally cultured (2D), as well as in self-assembled 3D ECM. From our overall raw data analysis, we observed that out of 249 targeted metabolites, the number of those robustly detected ranged from 127 to 155 across the samples with a mean value of 144. In Figure 2 and 3 the predicted metabolic pathways that are affected based on the raw data is shown in 2D (Figure 2) and 3D (Figure 3) systems. When we analyzed the metabolites that were up regulated by at least 2-fold in HKCs compared to HCKs a number of pathways that are involved in oxidative stress were affected. Examples are the citric acid cycle, the glycine-serine-threonine, the betaine and the glutathione metabolism. On the other hand, based on the metabolites where HCKs expressed in higher levels than HKCs, our software indicated significant regulation of pathways that are mainly involved in cell growth (biotin metabolism), toxicity protection (ammonia metabolism, and apoptosis prevention (ubiquinone biosynthesis). Based on these results we further analyzed the metabolites involved in oxidative stress.

Lactate: Cells and Constructs. Our system allows us to investigate the levels of various metabolites, both in cells that are conventionally cultured (2D), as well as in self-assembled 3D ECM. Lactate is produced from pyruvate via glycolysis and is an important metabolite that has been implicated in corneal edema. When lactate levels are high, corneal edema appears. High lactate levels are also a hallmark of many diseases and lactate has become a potential target for cancer therapies. Figure 4 shows a clear difference in lactate levels between cells cultured in monolayer (2D) and stratified cultures (3D). Lactate levels were significantly (p<0.05) higher when cells were allowed to develop their own ECM and grow in an environment that mimics the in vivo. HKC were significantly higher than both HCFs and HCKs. T3 stimulation only significantly affected HCFs (p<0.05) with a 2 fold up regulation of lactate levels. HCKs showed down regulation of lactate (p<0.05) with the same T3 treatment. Similarly, in the monolayer system, HCKs maintained the lowest levels of lactate when compared to both HCFs and HKCs.

Lactate/Malate ratio: Cells and Constructs. Lactate has been linked to oxidative stress and when paired with other specific metabolites, can act as an indicator of oxidative stress. One such combination is the lactate-malate ratio (L/M), which is known to be a critical regulator of oxidative stress. Malate is an intermediate in the citric acid cycle (or TCA cycle) produced from fumarate and an indicator of citric acid cycling. In vivo, the degree of elevation of the ratio and its timing are related to the severity of hypoxia and oxidative stress. In our systems, HCKs showed the lowest L/M ratio independent of condition and treatment (Figure 5), when compared to both HCFs (p<0.05) and HKs (p<0.01). In agreement with our lactate data, HKCs seem to be under more oxidative stress. This is enhanced in the 3D system where there is a significant 4 fold difference from the HCF (p<0.01). When HCFs were treated with
T3, L/M ratio was negatively affected showing a 2 and 4 fold up regulation in 2D (p<0.05) and 3D system (p<0.01) respectively. L/M ratio, on the other hand, was reduced in HKCs (p<0.05). Overall, HKCs abnormal levels of oxidative stress are enhanced in the 3D cultures, which is a system more accurately mirroring in vivo regulations (Figure 5). Lactate/malate can indicate the relative levels of glycolytic activity to TCA cycle activity.

**Lactate/Pyruvate ratio: Cells and Constructs.** Pyruvate represents the final step of glycolysis and a precursor to lactate. The relative ratio of lactate/pyruvate (L/P) has been used as a measure of anaerobic and aerobic corneal metabolism. However, the ratio also has been proposed as an indicator of oxidative stress. An increase in the L/P ratio has been reported to accompany corneal degradation and oxidative stress. Figure 6 shows ratio regulation on both our systems for all cell types. HCKs showed the lowest ratio of L/P in both systems and all conditions (p<0.001). HKC on the other hand displayed the greatest up regulation of L/P (p<0.0001) in the 3D model indicating oxidative stress. Upon T3 stimulation L/P levels were positively affected and significantly down regulated (p<0.001).

Figure 2 | Summary of pathway enrichment analysis in 2D system. Above is a display of the diversity of signaling pathways that are enriched on the basis of all the metabolites passing filtering criteria. The most significant p-values are in red while the least significant are in yellow and white. A) Shows pathways affected based on metabolites that were at least 2-fold down-regulated in HKCs compared to HCKs, B) Shows pathways affected based on metabolites that were at least 2-fold up-regulated in HKCs compared to HCKs.

Figure 3 | Summary of pathway enrichment analysis in 3D system. Above is a display of the diversity of signaling pathways that are enriched on the basis of all the metabolites passing filtering criteria. The most significant p-values are in red while the least significant are in yellow and white. A) Shows pathways affected based on metabolites that were at least 2-fold down-regulated in HKCs compared to HCKs, B) Shows pathways affected based on metabolites that were at least 2-fold up-regulated in HKCs compared to HCKs.
Consistent with our lactate results, the L/P levels were enhanced in the 3D model for the HCKs. No differences were seen for the HCFs which showed identical regulation on all systems and conditions.

GSH ratios: Cells and Constructs. Another way to measure levels of oxidative stress is by looking at a highly studied antioxidant and metabolite glutathione. Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. A decreased GSH-to-GSSG ratio is considered indicative of oxidative stress. In the keratoconic cornea, levels of GSH are low in central corneal buttons. In this study, we investigated the level of GSH-to-GSSG ratio in our 2D and 3D systems. (Figure 7) The lowest ratio of GSH-to-GSSG was detected in the HKCs (p<0.05) under all systems and conditions, indicating that the cells were and remained under oxidative stress. On the other hand, the highest ratio, not surprisingly, was seen in HCKs (p<0.05) indicating less oxidative stress levels. Unlike other indicators so far, such as lactate, L/M and L/P ratios (Figures 4, 5, and 6 respectively), the 3D system seemed to recover some of the HKCs stress levels, i.e under less oxidative stress. Figure 7 shows higher values of GSH-to-GSSG ratio for all three cell types in the 3D system compared to 2D (p<0.05). T3 treatment significantly increased the ratio values for HCKs (p<0.05) and HCFs (p<0.05), but not for HKCs. Overall, our data throughout showed that HKCs are distinct from both HCKs and HCFs and are under more oxidative stress regardless of which system is used (2D or 3D).

Arginine: Cells and Constructs. Arginine is another important metabolite that has been linked to oxidative stress. In the cornea, arginine is a key player in the overall immune privilege of the eye. Inhibition of arginine has been shown to accelerate graft rejection. We investigated the regulation of arginine in our systems and the results are shown in Figure 8. As expected, HCKs arginine levels were significantly higher than HCFs and HKCs (p<0.01), however that was only for the 2D system. In the 3D model, HCKs and HKCs showed similar levels where HCFs were significantly lower. This is another metabolite where its expression is regulated differently when the cells secrete their own ECM versus the conventional cell culture 2D model. While there is a clear indication that HKCs’ arginine levels are lower than the native HCKs, and therefore under oxidative stress, our data with T3 is somewhat confusing. In 2D, HCKs showed a massive down regulation of arginine, where HCFs and HKCs did not; where in 3D the effect was inverted with a big up regulation of arginine expression in HCKs. Clearly the role of T3 in oxidative stress regulation is not well understood and further characterization is currently under way.
stress, including keratoconus. Chwa and co-authors reported that the leakage of activated oxygen from mitochondria during the metabolic pathways of oxidative phosphorylation. The role of the mitochondria in energy metabolism and oxidative stress has been well established. It appears to be intimately linked to their generation of ROS that play a regulatory role in cellular metabolic processes. In fact, the majority of ROS are products of mitochondrial respiration. ROS production and oxidative stress have been linked with an array of pathologies, including type II diabetes, atherosclerosis, ischemia/reperfusion injury, and others.

Oxidative stress is defined as the imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to detoxify or repair any resulting damage from the reactive intermediates. In humans, oxidative stress depends on the size of these changes and whether the cell can overcome them and return to its original stage. One of the main sources of reactive oxygen is the leakage of activated oxygen from mitochondria during the metabolic pathway of oxidative phosphorylation. The role of the mitochondria in energy metabolism and oxidative stress has been well established.

The cornea is a transparent avascular tissue that absorbs approximately 80% of the incident ultraviolet B (UVB) light, making it highly sensitive and vulnerable to damage from free radicals and ROS. In the healthy cornea, there are a number of defensive mechanisms that are present to minimize and reduce the risk of oxidative damage. A variety of antioxidant enzymes are present such as catalase, glutathione peroxidase, and glutathione reductase. These enzymes work to neutralize ROS and prevent cellular damage from oxidative stress. This is in agreement with previous studies showing that GSH contribute to corneal degeneration in keratoconus. In order to ensure accuracy of our results we plotted ratios of GSH with other metabolites that are known to be a sign of oxidative stress such as malate and pyruvate. In all cases HKCs showed such levels suggesting that were under more stress than keratocytes.

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**Methods**

**Primary cultures of Human Corneal Keratocytes (HCK), Fibroblasts (HCF) and Keratoconus cells (HKC)**. HCKs and HCFs were isolated from human corneas from healthy patients without ocular disease. All samples were obtained from NDRI (National Disease Research Interchange, Philadelphia, PA). HKCs were isolated from healthy patients from patients with Keratoconus defects. These samples were obtained from Ula Jurkunas (Massachusetts Eye and Ear Infirmary, Boston, MA, USA) and Jospeh Hovindal (Aarhus University Hospital, Aarhus, Denmark). All research adhered to the tenets of the Declaration of Helsinki. Tissue was processed, as previously described. Briefly, corneal epithelium and endothelium were removed from the stroma by scraping with a razor blade. The stromal tissue was then cut into ~2 x 2 mm pieces and placed into T25 culture flasks. Explants were allowed to adhere to the bottom of the wells and then Eagle’s Minimum Essential Medium (EMEM: ATCC; Manassas, VA) with either 1% (HCKs) or 10% (HCFs and HKCs) fetal bovine serum (FBS: Atlantic Biologicals; Miami, FL) was added. Following 1–2 weeks of cultivation, the cells were passaged into 100 mm cell culture plates and allowed to grow to 100% confluence before being used in our two systems (2D and 3D).

**Conventional 2D cultures**. All cell types (HCKs, HCFs and HKCs) were cultured on conventional 6-well tissue culture plates and processed for qRT-PCR. Cells were seeded at 10^5 cells/well and cultured in EMEM with either 1% (HCKs) or 10% FBS...


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Author contributions

All authors contributed extensively to the work presented in this paper. D.K. and J.D.Z. designed the experiments and prepared the manuscript. D.K. executed the in vivo experiments, A.E.K.H. contributed to the manuscript preparation, C.B.R. executed and analyzed the RT-PCR experiment, V.T.R. helped interpreting the RT-PCR data and contributed to the manuscript preparation. J.M.A. executed the metabolomics experiments and contributed to the manuscript preparation.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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