Decreased Integrity, Content, and Increased Transcript Level of Mitochondrial DNA Are Associated with Keratoconus

Xiao-Dan Hao[1], Zhao-Li Chen[1], Ming-Li Qu[1], Xiao-Wen Zhao[1], Su-Xia Li[2], Peng Chen[1]*

1 State Key Laboratory Cultivation Base, Shandong Provincial Key Laboratory of Ophthalmology, Shandong Eye Institute, Shandong Academy of Medical Sciences, Qingdao, China, 2 Shandong Eye Hospital, Shandong Eye Institute, Shandong Academy of Medical Sciences, Jinan, China

* chenpeng599205@126.com

Abstract

Oxidative stress may play an important role in the pathogenesis of keratoconus (KC). Mitochondrial DNA (mtDNA) is involved in mitochondrial function, and the mtDNA content, integrity, and transcript level may affect the generation of reactive oxygen species (ROS) and be involved in the pathogenesis of KC. We designed a case-control study to research the relationship between KC and mtDNA integrity, content and transcription. One-hundred ninety-eight KC corneas and 106 normal corneas from Chinese patients were studied. Quantitative real-time PCR was used to measure the relative mtDNA content, transcript levels of mtDNA and related genes. Long-extension PCR was used to detect mtDNA damage. ROS, mitochondrial membrane potential and ATP were measured by respective assay kit, and Mito-Tracker Green was used to label the mitochondria. The relative mtDNA content of KC corneas was significantly lower than that of normal corneas ($P = 9.19 \times 10^{-24}$), possibly due to decreased expression of the mitochondrial transcription factor A (*TFAM*) gene ($P = 3.26 \times 10^{-3}$). In contrast, the transcript levels of mtDNA genes were significantly increased in KC corneas compared with normal corneas (NADH dehydrogenase subunit 1 [*ND1*]: $P = 1.79 \times 10^{-3}$; cytochrome c oxidase subunit 1 [*COX1*]: $P = 1.54 \times 10^{-3}$; NADH dehydrogenase subunit 1, [*ND6*]: $P = 4.62 \times 10^{-3}$). The latter may be the result of increased expression levels of mtDNA transcription-related genes mitochondrial RNA polymerase (*POLRMT*) ($P = 2.55 \times 10^{-4}$) and transcription factor B2 mitochondrial (*TFB2M*) ($P = 7.88 \times 10^{-5}$). KC corneas also had increased mtDNA damage ($P = 3.63 \times 10^{-10}$), higher ROS levels, and lower mitochondrial membrane potential and ATP levels compared with normal corneas. Decreased integrity, content and increased transcript level of mtDNA are associated with KC. These changes may affect the generation of ROS and play a role in the pathogenesis of KC.

Introduction

Keratoconus (KC) is a degenerative corneal disease, which is characterized by corneal ectasia, thinning, and cone-shaped protrusion, resulting in reduced vision, irregular astigmatism, and corneal scarring [1, 2]. It is a significant clinical problem worldwide, affecting both genders and all ethnicities [3, 4]. Owing to the limited availability of medical treatments, end-stage KC patients have to accept corneal transplantation.

The etiology of KC is poorly understood. Despite extensive research [4], the molecular pathogenesis of KC remains unknown in the majority of patients. Studies conducted in the early 1990s suggested that KC corneas suffered oxidative damage and that they had abnormal level of stress-related enzymes [5, 6], indicating that oxidative stress (OS) may play a role in the pathogenesis of KC [7, 8]. Oxidative phosphorylation in mitochondria is the major source of endogenous reactive oxygen species (ROS) [9]. Mitochondria have their own genome, mitochondrial DNA (mtDNA), which encodes 13 subunits of respiratory complexes I, III, IV, and V [10, 11]. As mtDNA is closely related with mitochondrial function, the mtDNA content, integrity, and transcript levels may affect the generation of ROS and be involved in the pathogenesis of KC. In a previous study, we showed that there was a significant decrease in the leukocyte mtDNA content of KC patients compared to that of control subjects [12]. In an American population, Atilano et al. reported that KC corneas had a lower mtDNA-to-nDNA (nuclear DNA) ratio and more mtDNA damage than do normal corneas [13]. These results suggest that mtDNA variations may be involved in the pathogenesis of KC, but as of yet no one had attempted to study the relationship between mtDNA and KC systematically in order to uncover the underlying mechanisms. Therefore, to further validate these results in larger cornea samples and study the underlying mechanisms, we carried out this study.

Hundreds to thousands of copies of mtDNA exist in each cell. Accumulating evidence has shown that mtDNA content control is an important aspect of mitochondrial genetics and biogenesis, and is essential for normal cellular function [14, 15]. In eukaryotic cells, mtDNA is replicated by mtDNA polymerase [16, 17]. The polymerase (DNA directed), gamma (*POLG*) gene (nDNA-encoded) encodes catalytic subunit of mtDNA polymerase, and plays a key role in mtDNA replication [18]. The mitochondrial transcription factor A (*TFAM*, nDNA-encoded) gene encodes a key mitochondrial transcription factor, which functions in mtDNA replication and regulates the mitochondrial genome content [19]. To further validate the relationship between mtDNA content and KC, we measured the relative mtDNA content and transcript levels of key genes (*POLG* and *TFAM*) related to mtDNA replication in KC and normal corneas.

Two promotors, heavy strand 2 and light strand, transcribe the entire heavy strand and light strand, respectively [20]. The process of transcription initiation in mitochondria involves three types of proteins: mitochondrial RNA polymerase (POLRMT, nDNA-encoded), TFAM (nDNA-encoded), and mitochondrial transcription factors B1 and B2 (TFB1M, TFB2M; nDNA-encoded) [21]. To investigate the relationship between mtDNA transcription and KC, and uncover the underlying reasons, we measured the transcript levels of genes located in the mtDNA light strand (NADH dehydrogenase subunit 6, *ND6;* mtDNA-encoded), heavy strand (NADH dehydrogenase subunit 1 (*ND1*, mtDNA-encoded) and cytochrome c oxidase subunit 1 (*COX1*, mtDNA-encoded)), as well as those of, key genes (*POLRMT*, *TFAM*, and *TFB2M;* nDNA-encoded) related to mtDNA transcription in the samples [22]. As the integrity of mtDNA is very important for mitochondrial function, and mtDNA damage is a source of OS, the levels of mtDNA damage in KC corneas were also examined.

In short, we designed a case-control study to research the relationship between mtDNA integrity, content, transcription, and KC comprehensively for the first time, in an attempt to

study the relationship between mtDNA and KC systematically and uncover the underlying mechanisms.

Materials and Methods

Ethics Statement

The study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Shandong Eye Institute (Qingdao, China). Written informed consent was obtained from all the participants (or guardians).

Samples

This study included 106 normal corneas (mean age, 38 years; range, 10–76) and 198 KC corneas (mean age, 21 years; range, 13–39). The surplus normal corneas after penetrating keratoplasty were obtained from the Eye Bank of Qingdao Eye Hospital. To ensure the consistency of the corneal regions studied in both groups, we just collected the normal corneas with larger area of surplus. The KC corneas were collected from ophthalmologists of Qingdao Eye Hospital and Shandong Eye Hospital after surgery. The diagnosis of KC was based on clinical examination (corneal stromal thinning, Vogt's striae, Fleischer's ring, Munson's sign, signs of videokeratography, and refractive errors). The KC and control corneas were harvested and handled in a similar manner. Three fresh normal corneas and three fresh KC corneas were used for cell culture immediately after surgery. Two fresh normal corneas and two fresh KC corneas were used for western blot analyses. In the remaining (including 101 normal corneas and 193 KC corneas), some of the corneas (37 normal corneas and 37 KC corneas) were cut into halves and stored under appropriate condition for DNA and RNA extraction respectively. The others corneas were stored at -80°C for DNA extraction immediately after surgery.

Cell culture and fluorescent staining

Normal human corneal fibroblast (HCF) and keratoconus corneal fibroblast (KCF) cells were isolated from three normal and three KC corneas as described previously [23], and cultured in DMEM/F12 medium (Corning, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) at 37°C with 5% CO_2. The cells were collected after 48 h of P1 generation for ROS, mitochondrial membrane potential, ATP measurement and mitochondria staining. The ROS levels were measured using an ROS Assay Kit (Beyotime, China). Mitochondrial membrane potential was measured using a JC-1 Assay Kit (Beyotime, China). ATP levels were measured using an ATP Assay Kit (Beyotime, China) and Mito-Tracker Green (Beyotime, China) was used to label mitochondria. All the fluorescent stainings were processed according to the manufacturer's protocols, and the results were observed using a laser scanning confocal microscope or an automatic microplate reader. For ROS, mitochondrial membrane potential and Mito-Tracker Green, each group was photographed, and the images were imported into Image J software (National Institutes of Health, Bethesda). The fluorescence intensity of each group was quantified and analyzed using Image J software.

Western blot

Total protein was prepared from each cornea using radioimmunoprecipitation assay (RIPA) buffer (Galen, Beijing, China) and quantified. Western blot analyses were performed as we described previously [24]. For each sample, the levels of proteins of interest were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primary antibodies included dynamin-related protein 1 (DRP1) antibody (ab184247, Abcam), superoxide dismutase 2

(SOD2) antibody (ab68155, Abcam), and GAPDH antibody (KC-5G5, Kangchen, Shanghai, China).

Measurement of mtDNA content

In total, 101 normal corneas and 193 KC corneas were processed for DNA extraction. Total DNA was isolated from the corneas using the standard phenol/chloroform method. The DNA concentration was measured by BioPhotometer (Eppendorf, Hamburg, Germany). The relative mtDNA content was measured by fluorescence-based quantitative real-time PCR (qPCR) and the $2^{-\Delta\Delta CT}$ method, as described in previous studies [12, 25]. The primer pair L375 (5′-CAC CAGCCTAACCAGATTTC-3′) and H475 (5′-GGGTTGTRTTGATGAGATTAGT-3′) was used for mtDNA detection. The amplification of the single copy nuclear b-globin gene (HBg1: 5′-GCTTCTGACACAACTGTG TTCACTAGC-3′; HBg2: 5′-CACCAACTTCATCCACGTT CACC-3′) was used for normalization. The values were expressed as mtDNA/nDNA ratios.

mtDNA transcript level and detection of the expression of related genes

In total, 37 normal corneas and 37 KC corneas were processed for RNA extraction and used to measure the transcript levels of mtDNA and related genes. Total RNA was isolated (NucleoS-pin RNA II System; Macherey-Nagel, Duren, Germany) and quantified using a spectropho-tometer (BioPhotometer; Eppendorf, Hamburg, Germany). cDNA was synthesized from RNA using a commercial kit (PrimeScript ™ RT Reagent Kit (Perfect Real Time); Takara, Dalian, China). The expression levels of *POLG* (nDNA-encoded), *TFAM* (nDNA-encoded), *ND6* (mtDNA-encoded), *ND1* (mtDNA-encoded), *COX1* (mtDNA-encoded), *POLRMT* (nDNA-encoded), and *TFB2M* (nDNA-encoded) genes were measured by qPCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The primer sequences of the genes used for qPCR are shown in Table 1.

Long Extension PCR (LX-PCR)

DNA samples from 101 normal corneas and 193 KC corneas were used to detect mtDNA dam-age. LX-PCR was performed as described previously with a few modifications [26]. The primer pair LXF (5′-TGAGGCCAAATATCATTCTGAGGGGC-3′) and LXR (5′-TTTCATCATGCGGA GATGTTGGATGG-3′) was used to amplify the mtDNA genome. Twenty microliter reactions contained 1× GC buffer I (Mg^{2+} Plus), 10 ng DNA, 0.4 µM each of the primers, 0.4 mM each of deoxynucleotide triphosphate, and 1 U of TaKaRa LA Taq DNA polymerase (Takara, Dalian, China). The reaction mixture was denatured at 95°C for three minutes followed by 35 cycles of 10 seconds at 98°C, 16 minutes at 68°C, and a final elongation for 10 minutes at 72°C. The LX-PCR products were separated by electrophoresis on a 0.8% agarose gel stained with ethidium bromide.

Table 1. Primer sequences of the genes used for qPCR.

Genes	Forward	Reverse
POLG	5′-CAACCCCTAGCTCTGACTGC-3′	5′-CCACGTCGTTGTAAGGTCCA-3′
TFAM	5′-CGCTCCCCCTTCAGTTTTGT-3′	5′-CCAACGCTGGGCAATTCTTC-3′
ND6	5′-TCAACGCCCATAATCATACAAA-3′	5′-GATGGCTATTGAGGAGTATCCTGAG-3′
ND1	5′-ACTACGCAAAGGTCCCAACG-3′	5′-GGCGGGTTTTAGGGGTTCTT-3′
COX1	5′-TTAGCTGACTCGCCACACTC-3′	5′-GGCCACCTACGGTGAAAAGA-3′
POLRMT	5′-GGGACCATCGAAAGGTGTCT-3′	5′-CTTCAGAACAGTGGCCCGAT-3′
TFB2M	5′-TCCCGGAAATCCAGACTTGT-3′	5′-TGATGACCAAGGCTCCATGTG-3′
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′	5′-TCCACCACCCTGTTGCTGTA-3′

doi:10.1371/journal.pone.0165580.t001

Statistical analysis

Previous studies reported an inverse association between the mtDNA content and age [27, 28], with mitochondrial dysfunction, mtDNA damage, and ROS increasing with age [29, 30]. An analysis of covariance was used to determine the differences in the relative mtDNA content and mtDNA damage of the KC and normal corneas, adjusting for the effect of age. A nonparametric test was used to determine the differences in transcript levels of mtDNA and related genes and the fluorescence intensity between the KC and normal corneas. Spearman's rank correlation analysis was used to evaluate the relationships between different parameters. All the statistical analyses were performed using SPSS 17.0 (SPSS Inc., USA). Statistical significance was defined as α = 0.05.

Results

KCF cells had increased ROS production, weaker mitochondrial fluorescence intensity, and decreased mitochondrial membrane potential, ATP levels

The fluorescence staining results of the normal and KCF cells are shown in Fig 1A–1E. The KCF cells had significantly higher ROS levels compared to the normal corneal fibroblast cells (Fig 1A and 1D). The fluorescence intensity of the Mito-Tracker-labeled mitochondria in the KCF cells was significantly weaker than that of the normal corneal fibroblast cells (Fig 1B and 1D). Similarly, the mitochondrial membrane potential and ATP levels of the KCF cells were significantly lower than that of the normal corneal fibroblast cells (Fig 1C–1E). These results showed that KCF cells had greater ROS production, lower mitochondrial fluorescence intensity, and lower mitochondrial membrane potential and ATP levels compared to normal corneal fibroblast cells. In addition, the western blot results showed that KC corneas have higher DRP1 expression and lower SOD2 expression compared to normal corneas (Fig 1F).

Decreased mtDNA content and expression of genes involved in replication in KC corneas

To test whether variations in the mtDNA content were associated with KC, we measured the mtDNA content in 193 KC corneas and 101 normal corneas. The results are shown in Table 2. There was a significant decrease of mtDNA content in KC corneas compared to normal corneas ($P = 9.19 \times 10^{-24}$). The medians and the 25th and 75th percentiles of relative mtDNA content of KC and normal corneas are shown in Fig 2.

To investigate the relationship between the replication-related genes *POLG* and *TFAM* and relative mtDNA content, the transcript levels of these genes were measured in 37 KC and 37 normal corneas. The results revealed no significant difference in the transcript level of the *POLG* gene between the KC corneas and normal corneas (Table 2, Fig 3). However, the *TFAM* transcript levels of the KC corneas were significantly lower than those of the normal corneas ($P = 3.26 \times 10^{-3}$) (Table 2, Fig 3). The *TFAM* transcript levels also showed a significant positive correlation with the mtDNA content (Correlation coefficient = 0.372, $P = 2.31 \times 10^{-3}$) (Table 3).

Increased transcript levels of mtDNA and transcription related genes in KC corneas

The transcript levels of *ND6* (locus in mtDNA light strand, mtDNA-encoded), *ND1* and *COX1* (locus in mtDNA heavy strand, mtDNA-encoded) were measured in 37 KC and 37 normal corneas. The mtDNA transcript levels of the transcription related genes *POLRMT* (nDNA-encoded) and *TFB2M* (nDNA-encoded) were also detected in the same samples. The results
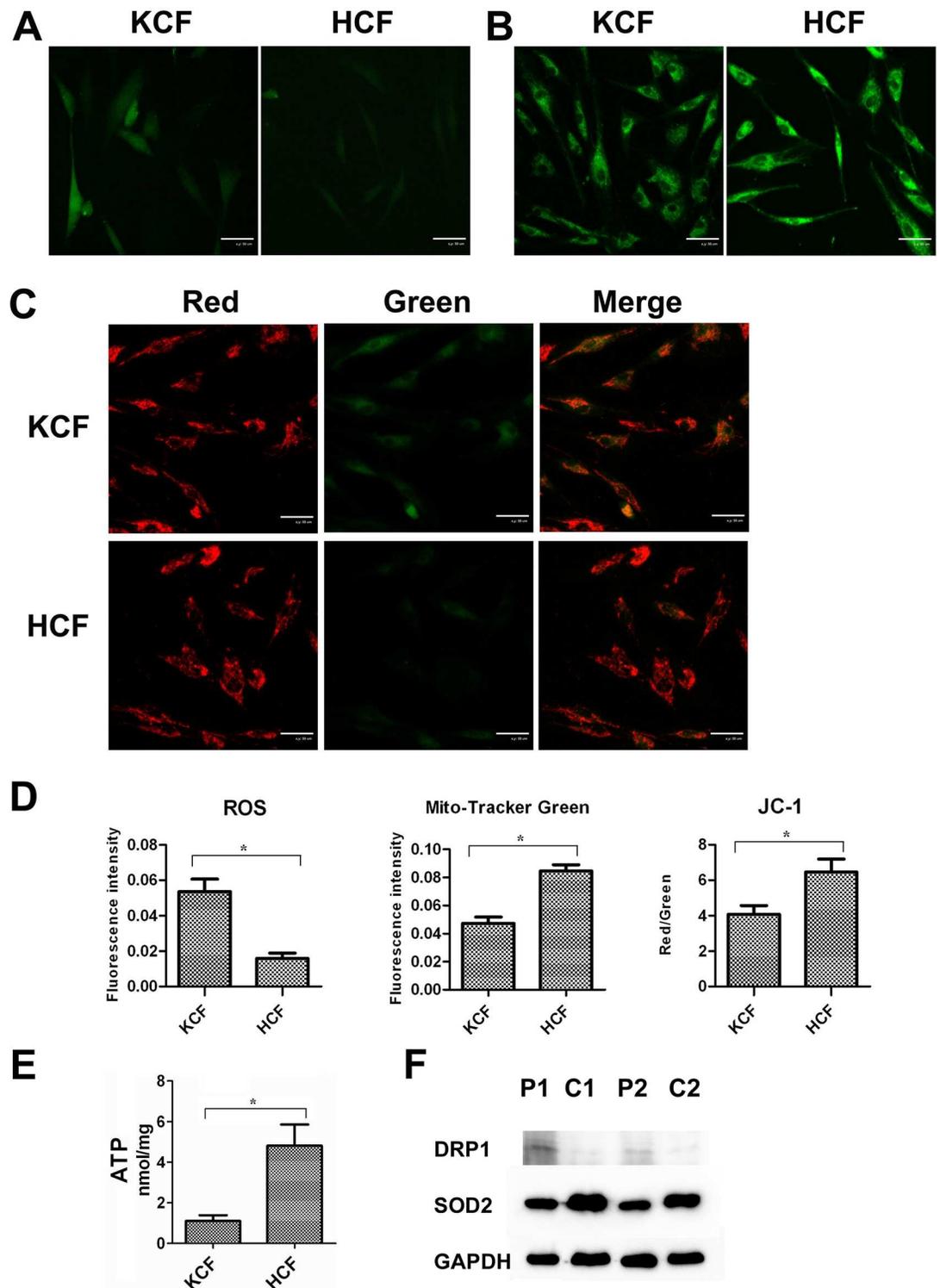
Fig 1. The inmunofluorescence staining results of HCF and KCF cells. A, reactive oxygen species (ROS); B, mitochondria labeled by Mito-Tracker Green; C, mitochondrial membrane potential measured by the JC-1 Assay Kit. Cells with high mitochondrial membrane potential showed enhanced red fluorescence. In contrast, cells with low mitochondrial membrane potential showed only green fluorescence. Differences in the ratio of red to green fluorescence are representative of changes in mitochondrial membrane depolarization. D, The fluorescence intensity of each group (n = 3) quantified by Image J software. E, ATP levels (n = 3); F, western blot analysis of DRP1 and SOD2. P1and P2: KC corneas; C1and C2: normal corneas.

doi:10.1371/journal.pone.0165580.g001

Table 2. Mitochondrial DNA content and expression of genes involved in mtDNA replication and transcription in the corneal samples of the patients and controls.

Parameters/Genes	Patients [a]	Controls [a]	P Value
mtDNA content			
Total	9.315±0.389	36.904±3.533	9.19×10^{-24}
Subgroup	5.238±0.238	72.605±4.955	1.62×10^{-17}
Transcript level of genes involved in mtDNA replication			
POLG	1.023±0.136	0.950±0.121	8.51×10^{-1}
TFAM	2.928±0.778	6.792±1.659	3.26×10^{-3}
Transcript level of L strand			
ND6	0.744±0.275	0.209±0.038	4.62×10^{-3}
Transcript level of H strand			
ND1	1.120±0.273	0.445±0.072	1.79×10^{-3}
COX1	1.179±0.210	0.609±0.094	1.54×10^{-3}
Transcript level of genes involved in mtDNA transcription			
POLRMT	1.173±0.102	0.641±0.077	2.55×10^{-4}
TFB2M	1.178±0.121	0.526±0.074	7.88×10^{-5}

Note. The values are given as means ±standard errors.

[a], including the 37 KC corneas and 37 normal corneas except the "total" group, which included 193 keratoconus corneas and 101 normal corneas.

doi:10.1371/journal.pone.0165580.t002

are shown in Table 2 and Fig 3. The *ND6* expression levels was significantly higher in the KC corneas than the normal corneas (Table 2, Fig 3). Similarly, the *ND1* and *COX1* expression levels of the KC corneas were significantly higher than those of normal corneas (Table 2, Fig 3). The transcript levels of *ND6*, *ND1* and *COX1* were all significantly negatively correlated with the mtDNA content (Table 3). The expression of the transcription related genes *POLRMT* and *TFB2M* was significantly increased in KC corneas compared to normal corneas (Table 2, Fig 3), and the expression was significantly positively correlated with the transcript levels of mtDNA (Table 3, all *P* values $<1 \times 10^{-3}$).

Increased mtDNA Damage in KC Corneas

LX-PCR was used to detect mtDNA damage, as previous described. The major LX-PCR product representing full-length mtDNA is 16.2 kb. When nucleotide substitutions, rearrangements, or deletions are present in the mtDNA template, LX-PCR yields smaller-sized products. We did LX-PCR analysis in 193 KC corneas and 101 normal corneas. Smaller-sized mtDNA bands (<16.2 kb and >1 kb) in the KC and normal samples were quantified. The mean number of smaller-sized mtDNA bands in the KC and normal corneas was 4.660 (standard error (SE) = 0.176) and 2.634 (SE = 0.182), respectively. There was a significant increase in the number of smaller-sized mtDNA bands in the KC corneas compared to the normal corneas ($P = 3.63 \times 10^{-10}$). The medians and the 25th and 75th percentiles of the smaller-sized mtDNA bands of KC and normal corneas are shown in Fig 4. The smaller-sized mtDNA bands also showed a significant negative correlation with the mtDNA content (correlation coefficient = -0.289, $P = 5.70 \times 10^{-7}$). For the subgroup (i.e., the 37 KC and 37 normal corneas), the mean number of smaller-sized mtDNA bands in the KC and normal corneas was 4.972 (SE = 0.426) and 2.417 (SE = 0.212), respectively. There was a significant increase in the number of smaller-sized mtDNA bands in the KC corneas compared to the normal corneas in the subgroups ($P = 4.82 \times 10^{-5}$). According to the subgroup data, the smaller-sized mtDNA bands showed a significant positive correlation with mtDNA transcription (Table 3).These results showed that
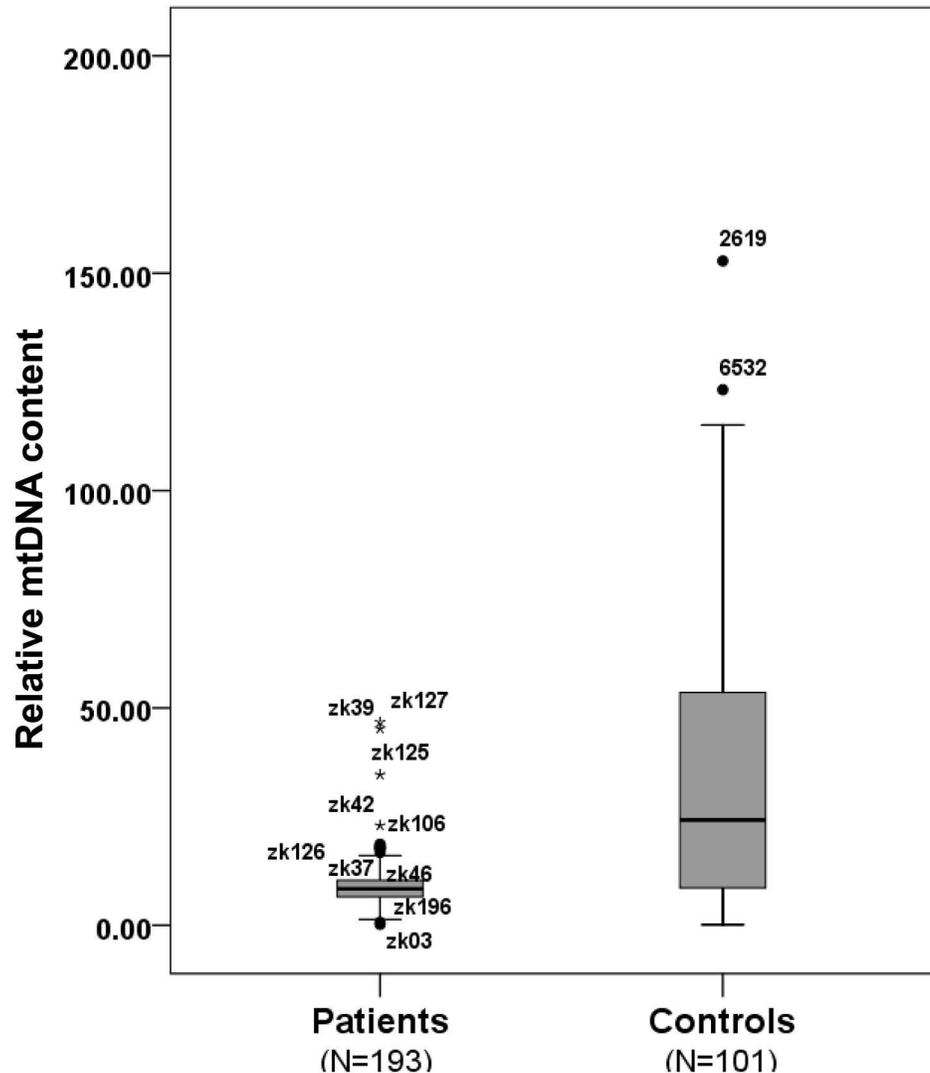
Fig 2. Relative mtDNA content in 101 normal corneas and 193 KC corneas. The medians and the 25th and 75th percentiles of relative mtDNA content in the KC and normal corneas are shown.

doi:10.1371/journal.pone.0165580.g002

the KC corneas had significantly decreased mtDNA content and increased mtDNA transcription and damage relative to normal corneas.

Discussion

Previous studies found that KCF cells had increased ROS and lower mitochondrial membrane potential compared with normal corneas [7, 8, 31], which indicated that these components may play a part in the pathogenesis of KC [32]. To validate these results, we repeated the experiments performed in those studies using KC and normal corneas and obtained consistent findings. In the present study, KCF cells had more ROS products, and lower mitochondrial membrane potential and ATP levels compared with normal corneal fibroblasts, suggesting that mitochondrial function plays a role in the pathogenesis of KC. In addition, we also found that KC corneas have higher DRP1 expression and lower SOD2 expression than normal corneas. DRP1 is a fundamental component of mitochondrial fission, and fission results in fragmented
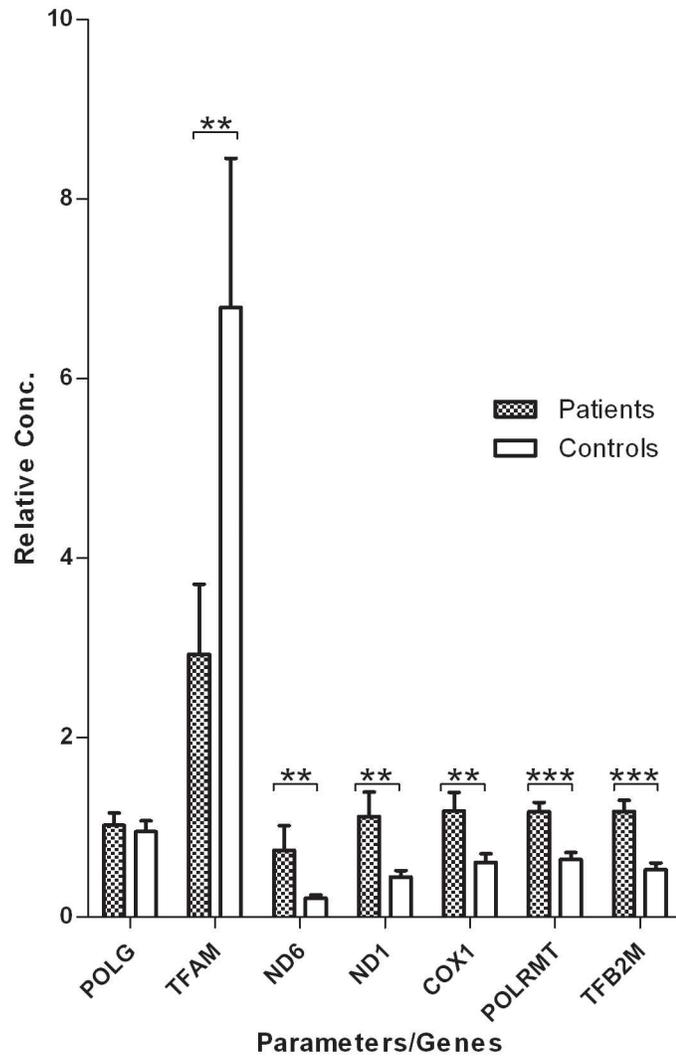
Fig 3. The expression of genes involved in mtDNA replication and transcription in each of the 37 corneal samples of the patients and controls. **, *P*<0.01; ***, *P*<0.001.

doi:10.1371/journal.pone.0165580.g003

Table 3. Spearman's correlation coefficients between the mtDNA content, damage and genes involved in mtDNA replication and transcription in the corneal samples of the patients and controls.

Correlation coefficient	mtDNA content	POLG	TFAM	ND6	ND1	COX1	POLRMT	TFB2M	mtDNA damage
mtDNA content	1.000	0.018	0.372**	-0.346**	-0.332**	-0.363**	-0.349**	-0.519***	-0.289***
ND6	-0.346**	0.077	-0.274*	1.000	0.815***	0.797***	0.672***	0.730***	0.366**
ND1	-0.332**	0.109	-0.156	0.815***	1.000	0.946***	0.716***	0.724***	0.443***
COX1	-0.363**	0.155	-0.147	0.797***	0.946***	1.000	0.723***	0.746***	0.390***

Note.

*, *P*<0.05;

**, *P*<0.01;

***, *P*<0.001.

doi:10.1371/journal.pone.0165580.t003
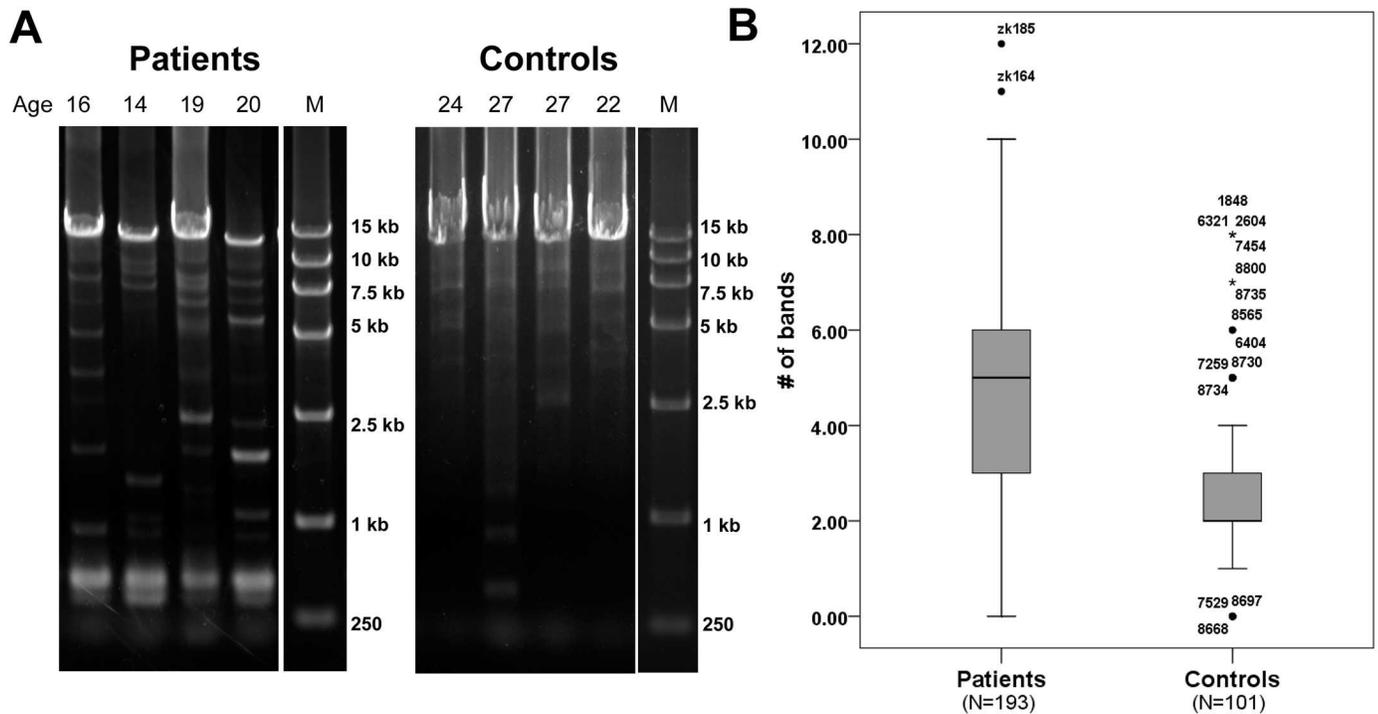
Fig 4. Increased mtDNA damage in KC corneas. A, Representative agarose gels image of mtDNA damage in the KC patients and controls. B, Quantitation of smaller-sized bands per individual in normal and KC corneal LX-PCR mtDNA. The medians and the 25th and 75th percentiles of smaller-sized mtDNA bands of the KC and normal corneas are shown.

mitochondria more capable of producing of ROS [33]. SOD2 is a member of the iron/manganese superoxide dismutase family, and plays an antiapoptotic role against oxidative stress [34]. Higher DRP1 expression and lower SOD2 expression in KC corneas verified again that the oxidative stress is involved in KC, and plays an important role in the pathogenesis of KC.

Accumulating evidence has shown that mtDNA content control is very important for mitochondrial biogenesis and normal cellular function [14, 15]. Previous studies observed that the mtDNA content is associated with many eye diseases other than KC, including autosomal dominant optic atrophy, diabetic retinopathy, and Leber's hereditary optic neuropathy [35–37]. Atilano et al. found that KC corneas (N = 16) had lower mtDNA-to-nDNA ratios than normal corneas (N = 10) [13]. However, their results showed this trend with an insignificant P value ($P < 0.7$). In the present study, we measured the relative mtDNA content in a larger sample size and found a significant decrease of mtDNA content in KC corneas compared to normal corneas. This result suggested that decreased mtDNA content is associated with KC. In the present study, there was no significant difference in *POLG* expression levels between the KC and normal corneas, but that *TFAM* expression levels of the KC corneas were significantly lower than those of the normal corneas. The *TFAM* expression levels also showed a significant positive correlation with the mtDNA content. The findings suggest that the lower relative mtDNA content of KC corneas may be due to decreased *TFAM* gene expression.

The transcript level of mtDNA is crucial for the function of the oxidative phosphorylation. Chwa et al. reported that the mitochondrial cytochrome oxidase subunit 2 RNA levels were increased in the KC fibroblasts cultured at pH 7 compared with the normal cultures, and mitochondrial cytochrome oxidase subunit 1 RNA levels were elevated in KC cells but without reaching significance [31]. In the current study, we further found that the transcript levels of

mtDNA were significantly increased in KC corneas compared with normal corneas, and the expressions of the transcription-related genes *POLRMT* and *TFB2M* were significantly increased in KC corneas and positively correlated with the transcript levels of mtDNA. These results suggested that an increased transcript level of mtDNA is associated with KC, which may be due to increased *POLRMT* and *TFB2M* gene expression.

The integrity of mtDNA is very important for mitochondrial function, and mtDNA damage is a source of OS. The human cornea is exposed to oxidizing UV and blue light, and contains the highest amount of mtDNA fragment deletions compared to other tissues of the eye [38, 39]. Increased mtDNA damage is reported to be involved in many OS related eye diseases, including age-related macular degeneration, diabetic retinopathy, age-related cataracts, and KC [13, 32, 40–42]. OS can cause DNA damage [43]. As a major producer of ROS, mitochondria may thus be subjected to direct attack by ROS [44]. mtDNA is particularly prone to oxidative damage compared to nuclear DNA; because of its proximity to the ROS-generating respiratory chain, it is directly exposed to ROS and inefficient DNA repair systems [45]. Conversely, mtDNA damage is also a source of OS. mtDNA damage could lead to progressive respiratory chain dysfunction and a further increase in ROS production as a consequence of this dysfunction [43]. Atilano et al. reported that KC corneas (N = 16) exhibit more mtDNA damage than do normal corneas (N = 10) in the American KC population [13]. To further validate this result in Chinese population, we detected the mtDNA damage in a larger sample size, and found that there was a significant increase of mtDNA damage in KC corneas compared to normal corneas. This result suggested that increased mtDNA damage is associated with KC.

In summary, the following results were obtained in this study. The mtDNA content of KC corneas was lower than that of normal corneas, possibly due to decreased *TFAM* gene expression. However, the transcript levels of mtDNA were significantly increased in KC patients corneas compared to normal corneas. In addition, the expression levels of the transcription related genes *POLRMT* and *TFB2M* were significantly increased in KC patients' corneas, and they were positively correlated with the transcript levels of mtDNA. KC corneas also showed more mtDNA damage, higher ROS levels, and lower mitochondrial membrane potential and ATP levels compared to normal corneas. mtDNA damage showed a significant negative correlation with mtDNA content. In addition, mtDNA transcript levels were significantly negatively correlated with mtDNA content and positively correlated with mtDNA damage. Based on the results of the present study, we speculate that the mechanism underlying the role of mtDNA in KC may proceed as follows: KC corneas have higher transcript level of mtDNA, possibly due to increased expression of the *POLRMT* and *TFB2M* genes. A combination of higher transcript level of mtDNA and higher mtDNA damage could lead to progressive respiratory chain dysfunction and a further increase in ROS production as a consequence of this dysfunction [32]. Decreased mtDNA content caused by reduced *TFAM* gene expression may lead to increased proportion of mtDNA damage and reduced quantities of normal mtDNA in the corneal stroma, further increasing ROS formation and additional OS in cornea. However, this speculation needs further validation and a detailed assay of cornea samples, such as Northern blots of mtDNA-transcribed mRNAs or other functional analysis.

Conclusions

In conclusion, we found that decreased integrity, content and increased transcript level of mtDNA is associated with KC, and these changes may affect the generation of ROS and be involved in the pathogenesis of KC.

Supporting Information

S1 Table. Relative mtDNA content in the 193 KC corneas and 101 normal corneas.
(XLS)

S2 Table. Expression of genes involved in mtDNA replication and transcription in corneal samples of the patients and controls.
(XLS)

S3 Table. Smaller-sized mtDNA bands in 193 KC corneas and 101 normal corneas.
(XLS)

Acknowledgments

We are greatly indebted to the individuals who participated in this research.

Author Contributions

Conceptualization: XDH PC.

Formal analysis: XDH PC.

Funding acquisition: XDH PC.

Investigation: XDH ZLC MLQ XWZ.

Methodology: XDH PC.

Resources: ZLC MLQ SXL.

Writing – original draft: XDH.

Writing – review & editing: XDH PC.

References

1. Krachmer JH, Feder RS and Belin MW. Keratoconus and related noninflammatory corneal thinning disorders. Surv Ophthalmol 1984; 28(4): 293–322. PMID: 6230745

2. Rabinowitz YS. Keratoconus. Surv Ophthalmol 1998; 42(4): 297–319. PMID: 9493273

3. Kok YO, Tan GF and Loon SC. Review: keratoconus in Asia. Cornea 2012; 31(5): 581–593. doi: 10.1097/ICO.0b013e31820cd61d PMID: 22314815

4. Rahman I, Carley F, Hillarby C, Brahma A and Tullo AB. Penetrating keratoplasty: indications, outcomes, and complications. Eye (Lond) 2009; 23(6): 1288–1294.

5. Gondhowiardjo TD and van Haeringen NJ. Corneal aldehyde dehydrogenase, glutathione reductase, and glutathione S-transferase in pathologic corneas. Cornea 1993; 12(4): 310–314. PMID: 8339559

6. Gondhowiardjo TD, van Haeringen NJ, Volker-Dieben HJ, Beekhuis HW, Kok JH, van Rij G, et al. Analysis of corneal aldehyde dehydrogenase patterns in pathologic corneas. Cornea 1993; 12(2): 146–154. PMID: 8500322

7. Behndig A, Karlsson K, Johansson BO, Brannstrom T and Marklund SL. Superoxide dismutase isoenzymes in the normal and diseased human cornea. Invest Ophthalmol Vis Sci 2001; 42(10): 2293–2296. PMID: 11527942

8. Chwa M, Atilano SR, Reddy V, Jordan N, Kim DW and Kenney MC. Increased stress-induced generation of reactive oxygen species and apoptosis in human keratoconus fibroblasts. Invest Ophthalmol Vis Sci 2006; 47(5): 1902–1910. doi: 10.1167/iovs.05-0828 PMID: 16638997

9. Nohl H. Generation of superoxide radicals as byproduct of cellular respiration. Ann Biol Clin (Paris) 1994; 52(3): 199–204.

10. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature 1981; 290(5806): 457–465. PMID: 7219534

11. Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM and Howell N. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet 1999; 23(2): 147. doi: 10.1038/13779 PMID: 10508508

12. Hao XD, Chen P, Wang Y, Li SX and Xie LX. Mitochondrial DNA copy number, but not haplogroup is associated with keratoconus in Han Chinese population. Exp Eye Res 2015; 132(59–63). doi: 10.1016/j.exer.2015.01.016 PMID: 25613073

13. Atilano SR, Coskun P, Chwa M, Jordan N, Reddy V, Le K, et al. Accumulation of mitochondrial DNA damage in keratoconus corneas. Invest Ophthalmol Vis Sci 2005; 46(4): 1256–1263. doi: 10.1167/iovs.04-1395 PMID: 15790887

14. Clay Montier LL, Deng JJ and Bai Y. Number matters: control of mammalian mitochondrial DNA copy number. J Genet Genomics 2009; 36(3): 125–131. doi: 10.1016/S1673-8527(08)60099-5 PMID: 19302968

15. Gaziev AI, Abdullaev S and Podlutsky A. Mitochondrial function and mitochondrial DNA maintenance with advancing age. Biogerontology 2014; 15(5): 417–438. doi: 10.1007/s10522-014-9515-2 PMID: 25015781

16. McKinney EA and Oliveira MT. Replicating animal mitochondrial DNA. Genet Mol Biol 2013; 36(3): 308–315. doi: 10.1590/S1415-47572013000300002 PMID: 24130435

17. Kaguni LS. DNA polymerase gamma, the mitochondrial replicase. Annu Rev Biochem 2004; 73(293–320). doi: 10.1146/annurev.biochem.72.121801.161455 PMID: 15189144

18. Ropp PA and Copeland WC. Cloning and characterization of the human mitochondrial DNA polymerase, DNA polymerase gamma. Genomics 1996; 36(3): 449–458. doi: 10.1006/geno.1996.0490 PMID: 8884268

19. Campbell CT, Kolesar JE and Kaufman BA. Mitochondrial transcription factor A regulates mitochondrial transcription initiation, DNA packaging, and genome copy number. Biochim Biophys Acta 2012; 1819(9–10): 921–929. doi: 10.1016/j.bbagrm.2012.03.002 PMID: 22465614

20. Montoya J, Christianson T, Levens D, Rabinowitz M and Attardi G. Identification of initiation sites for heavy-strand and light-strand transcription in human mitochondrial DNA. Proc Natl Acad Sci U S A 1982; 79(23): 7195–7199. PMID: 6185947

21. Asin-Cayuela J and Gustafsson CM. Mitochondrial transcription and its regulation in mammalian cells. Trends Biochem Sci 2007; 32(3): 111–117. doi: 10.1016/j.tibs.2007.01.003 PMID: 17291767

22. Litonin D, Sologub M, Shi Y, Savkina M, Anikin M, Falkenberg M, et al. Human mitochondrial transcription revisited: only TFAM and TFB2M are required for transcription of the mitochondrial genes in vitro. J Biol Chem 2010; 285(24): 18129–18133. doi: 10.1074/jbc.C110.128918 PMID: 20410300

23. Zhou Q, Wang Y, Yang L, Chen P, Dong X and Xie L. Histone deacetylase inhibitors blocked activation and caused senescence of corneal stromal cells. Mol Vis 2008; 14(2556–2565. PMID: 19122829

24. Chen P, Dai Y, Wu X, Wang Y, Sun S, Xiao J, et al. Mutations in the ABCA3 gene are associated with cataract-microcornea syndrome. Invest Ophthalmol Vis Sci 2014; 55(12): 8031–8043. doi: 10.1167/iovs.14-14098 PMID: 25406294

25. Bi R, Zhang AM, Zhang W, Kong QP, Wu BL, Yang XH, et al. The acquisition of an inheritable 50-bp deletion in the human mtDNA control region does not affect the mtDNA copy number in peripheral blood cells. Hum Mutat 2010; 31(5): 538–543. doi: 10.1002/humu.21220 PMID: 20151402

26. Jessie BC, Sun CQ, Irons HR, Marshall FF, Wallace DC and Petros JA. Accumulation of mitochondrial DNA deletions in the malignant prostate of patients of different ages. Exp Gerontol 2001; 37(1): 169–174. PMID: 11738157

27. He YH, Lu X, Wu H, Cai WW, Yang LQ, Xu LY, et al. Mitochondrial DNA content contributes to healthy aging in Chinese: a study from nonagenarians and centenarians. Neurobiol Aging 2014.

28. Mengel-From J, Thinggaard M, Dalgard C, Kyvik KO, Christensen K and Christiansen L. Mitochondrial DNA copy number in peripheral blood cells declines with age and is associated with general health among elderly. Hum Genet 2014; 133(9): 1149–1159. doi: 10.1007/s00439-014-1458-9 PMID: 24902542

29. Hayakawa M, Hattori K, Sugiyama S and Ozawa T. Age-associated oxygen damage and mutations in mitochondrial DNA in human hearts. Biochem Biophys Res Commun 1992; 189(2): 979–985. PMID: 1472070

30. Hudson EK, Hogue BA, Souza-Pinto NC, Croteau DL, Anson RM, Bohr VA, et al. Age-associated change in mitochondrial DNA damage. Free Radic Res 1998; 29(6): 573–579. PMID: 10098461

31. Chwa M, Atilano SR, Hertzog D, Zheng H, Langberg J, Kim DW, et al. Hypersensitive response to oxidative stress in keratoconus corneal fibroblasts. Invest Ophthalmol Vis Sci 2008; 49(10): 4361–4369. doi: 10.1167/iovs.08-1969 PMID: 18515575

32. Wojcik KA, Kaminska A, Blasiak J, Szaflik J and Szaflik JP. Oxidative stress in the pathogenesis of keratoconus and Fuchs endothelial corneal dystrophy. Int J Mol Sci 2013; 14(9): 19294–19308. doi: 10.3390/ijms140919294 PMID: 24065107

33. Archer SL. Mitochondrial dynamics—mitochondrial fission and fusion in human diseases. N Engl J Med 2013; 369(23): 2236–2251. doi: 10.1056/NEJMra1215233 PMID: 24304053

34. Flynn JM and Melov S. SOD2 in mitochondrial dysfunction and neurodegeneration. Free Radic Biol Med 2013; 62(4–12). doi: 10.1016/j.freeradbiomed.2013.05.027 PMID: 23727323

35. Kim JY, Hwang JM, Ko HS, Seong MW, Park BJ and Park SS. Mitochondrial DNA content is decreased in autosomal dominant optic atrophy. Neurology 2005; 64(6): 966–972. doi: 10.1212/01.WNL.0000157282.76715.B1 PMID: 15781809

36. Santos JM, Tewari S, Goldberg AF and Kowluru RA. Mitochondrial biogenesis and the development of diabetic retinopathy. Free Radic Biol Med 2011; 51(10): 1849–1860. doi: 10.1016/j.freeradbiomed.2011.08.017 PMID: 21911054

37. Giordano C, Iommarini L, Giordano L, Maresca A, Pisano A, Valentino ML, et al. Efficient mitochondrial biogenesis drives incomplete penetrance in Leber's hereditary optic neuropathy. Brain 2014; 137(Pt 2): 335–353. doi: 10.1093/brain/awt343 PMID: 24369379

38. Gendron SP, Bastien N, Mallet JD and Rochette PJ. The 3895-bp mitochondrial DNA deletion in the human eye: a potential involvement in corneal ageing and macular degeneration. Mutagenesis 2013; 28(2): 197–204. doi: 10.1093/mutage/ges071 PMID: 23408842

39. Gendron SP, Mallet JD, Bastien N and Rochette PJ. Mitochondrial DNA common deletion in the human eye: a relation with corneal aging. Mech Ageing Dev 2012; 133(2–3): 68–74. doi: 10.1016/j.mad.2012.01.002 PMID: 22285291

40. Lin H, Xu H, Liang FQ, Liang H, Gupta P, Havey AN, et al. Mitochondrial DNA damage and repair in RPE associated with aging and age-related macular degeneration. Invest Ophthalmol Vis Sci 2011; 52(6): 3521–3529. doi: 10.1167/iovs.10-6163 PMID: 21273542

41. Kowluru RA and Mishra M. Oxidative stress, mitochondrial damage and diabetic retinopathy. Biochim Biophys Acta 2015; 1852(11): 2474–2483. doi: 10.1016/j.bbadis.2015.08.001 PMID: 26248057

42. Tinaztepe OE, Ay M and Eser E. Nuclear and Mitochondrial DNA of Age-Related Cataract Patients Are Susceptible to Oxidative Damage. Curr Eye Res 2016: 1–6.

43. Shokolenko I, Venediktova N, Bochkareva A, Wilson GL and Alexeyev MF. Oxidative stress induces degradation of mitochondrial DNA. Nucleic Acids Res 2009; 37(8): 2539–2548. doi: 10.1093/nar/gkp100 PMID: 19264794

44. Murphy MP. How mitochondria produce reactive oxygen species. Biochem J 2009; 417(1): 1–13. doi: 10.1042/BJ20081386 PMID: 19061483

45. Salazar JJ and Van Houten B. Preferential mitochondrial DNA injury caused by glucose oxidase as a steady generator of hydrogen peroxide in human fibroblasts. Mutat Res 1997; 385(2): 139–149. PMID: 9447235