Intravitreal Injection of Mitochondrial DNA Induces Cell Damage and Retinal Dysfunction in Rats

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Research

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Abstract

Background: Retinal neurodegeneration is induced by a variety of environmental insults and stresses, but the exact mechanisms are unclear. In the present study, we explored the involvement of cytosolic mitochondrial DNA (mtDNA), resulting in the cGAS-STING dependent inflammatory response and apoptosis in retinal damage in vivo.

Methods: Retinal injury was induced by white light or intravitreal injection of lipopolysaccharide (LPS). After light- or LPS-induced injury, the amount of cytosolic mtDNA in the retina was detected by PCR. The mtDNA was isolated and used to transf ect retinas in vivo. WB and real-time PCR were used to evaluate the activation of cGAS-STING pathway and the levels of apoptosis-associated protein at different times after mtDNA stimulation. Retinal cell apoptosis rate was detected by TUNEL staining. Full-field electroretinography (ERG) was used to assess the retinal function.

Results: Light injury and the intravitreal injection of LPS both caused the leakage of mtDNA into the cytoplasm in retinal tissue. After the transfection of mtDNA in vivo, the levels of cGAS, STING, and IFN-β mRNAs and the protein levels of STING, phospho-TBK1, phospho-IRF3, and IFN-β were upregulated. mtDNA stimulation also induced the phosphorylation of caspase 3 and caspase 9. BAX and BAK were increased at both the mRNA and protein levels. The release of cytochrome c from the mitochondria to the cytosol was increased after mtDNA stimulation. The wave amplitudes on ERG decreased and retinal cell apoptosis was detected after mtDNA stimulation.

Conclusion: Cytosolic mtDNA triggers an inflammatory response. It also promotes apoptosis and the dysfunction of the retina.

Background

Retina is a sophisticated architecture in the eye. It is highly differentiated and metabolic demand to maintain the homeostasis of all retinal cell types and support healthy vision[1]. The retinal cells have to response to a variety of environmental insults and stresses, including age, light-induced damage, oxidative stress, and inherited mutations[1]. Retinal degeneration that can be caused by numerous mechanisms occurs in a group of clinical eye diseases characterized by the progressive loss of vision [2]. Among these mechanisms the degeneration and death of photoreceptors can result in many disorders[1, 3]. The extensive loss of photoreceptors, leaving a deafferented neural retina, is followed by extensive remodeling and ultimately by neurodegeneration[4]. Numerous studies have addressed retinal neurodegeneration, but the exact mechanism is still unclear. The previous studies of our team found that light-injury and intravitreal LPS injection can lead to the inflammatory response or apoptosis in rats’ retina[5, 6]. Exposure to bright light upregulated the activation of caspase-3 and caspase-9, which caused the apoptosis of photoreceptor cells and reduced the function of retina[7]. In the lipopolysaccharide-induced retinal inflammation in rats, we also found the increase of inflammation-related factors[8].
Mitochondria have increasingly been implicated as the gatekeepers of cell fate, with decisive roles in diverse cellular responses, including apoptosis, autophagy, and innate immunity\[9, 10\]. Mitochondria facilitate the innate immune response to infection and injury by releasing mitochondrial DNA (mtDNA), which is recognized as a damage-associated molecular pattern (DAMP) by the cell’s innate immune components\[11\]. Various researchers have shown that cytosolic mtDNA drives the activation of the noncanonical inflammasome in retinal pigment epithelial (RPE) cells and induces cell damage\[12, 13\]. We previously reported that pathological stimuli lead to the release of mtDNA into the cytosol in retinal vascular endothelial cells in vitro and that cytosolic mtDNA is recognized by the DNA sensor cyclic GMP-AMP synthase (cGAS), which increases the expression of inflammatory cytokines through the STING–TBK1 signaling pathway\[14\]. In the present study, we further explored the retinal damage caused by cytosolic mtDNA in vivo.

**Methods**

**Animals and Induction of Retinal Injury by Light or Lipopolysaccharide (LPS)**

The animal protocols used in the study were approved by the Animal Ethics Committee of the Eye and Ear Nose Throat Hospital of Fudan University, Shanghai, China and the experiments complied with the Association for Research in Vision and Ophthalmology’s Statement on the Use of Animals in Research. Sprague–Dawley rats (male, 6–8 weeks old, approximately 200 g) were kept in a colony room on a 12-h light/12-h dark cycle at 22–24 °C. Normal food and water were available ad libitum. All experiments were performed on the animals after an intraperitoneal injection of 10% chloral hydrate (0.4 ml/100 g). At the end of the experiments, the animals were anesthetized with an overdose of 10% chloral hydrate and killed by cervical dislocation. All operations were performed in such a way as to minimize animal suffering. Before light exposure, the animals’ pupils were dilated with 1% atropine eye drops (Santen Pharmaceuticals Co., Ltd, Osaka, Japan). In the light-injury model, the rats were separated into individual transparent boxes with wire tops and exposed continuously to bright light (5000 lux) in a light box to induce retinal degeneration. After exposure for 24 h, the rats were returned to the normal light/dark cycle and room conditions. In the intravitreal LPS injection model of retinal damage, phosphate-buffered saline (PBS; HyClone, Logan, UT, USA) was used to dissolve and dilute LPS (Sigma-Aldrich, St. Louis, MO, USA) solution to a concentration of 125 ng/μl. Rats were anesthetized and the pupils were dilated with atropine sulfate (Santen Pharmaceuticals Co., Ltd). Then a Hamilton microinjector (Hamilton, Reno, NV, USA) was used to perform the intravitreal injection with LPS (2 μl, 125 ng/μl) at the 1 mm posterior to the limbus. The right retinal samples were collected before exposure or 1, 3, 5, and 7 days after light- or LPS-induced injury for further analysis.

**Isolation of Cytosolic and Mitochondrial Fractions and Detection of Cytosolic mtDNA**

The anterior segment of each eye was removed and the retina was isolated after the eye had been enucleated after light- or LPS-induced injury, according to the experimental schedule. The retinas were weighed and cut up. Each fresh retina (usually ≤ 1 h after the animal was killed) was processed with the
Mitochondria Isolation Kit for Tissue (C3606, Beyotime, Shanghai, China). The tissue was then mixed with 10 times the volume of mitochondrial separating reagent A and homogenized with a Dounce tissue grinder (10 passes). The homogenates were centrifuged at 600 × g for 10 min at 4 °C. The supernatants were pipetted, transferred to fresh 1.5 ml tubes, and centrifuged at 11,000 × g for 10 min at 4 °C. The mitochondrial DNA was isolated from the cytosolic supernatant. All experimental procedures were performed on ice. The PCR assay was used to detect the cytosolic mtDNA, as described in our previous work[14]. In brief, the above-mentioned mtDNA was mixed with Buffer FG1 and Buffer FG2 of the FlexiGene DNA Kit (no. 51206, Qiagen) in a 2 ml tube. After inverting the tube three times, the mixture was incubated at 65 °C for 10 min. Isopropanol (100%) was used to induce DNA precipitation. The tube was centrifuged for 3 min at 10,000 × g. Then Buffer FG3 was used to dissolve the DNA precipitation at 65 °C for 30 min. Quantitative PCR (qPCR) was used to amplify the sequence encoding mitochondrial cytochrome c oxidase 1 (mt-Co1) to detect the mtDNA and to amplify the 18SrDNA sequence to detect the nuclear DNA. The levels of mtDNA were normalized to the nuclear DNA and compared between groups. Primer sequences are listed in Supplementary Table S1.

**Preparation and Retinal Transfection of mtDNA**

The mtDNA was isolated from normal rat retinal cells as previously described[14]. The rat retinal microvascular endothelial cells were collected, washed with ice-cold PBS, and resuspended in 1 × Cytosol Extraction Buffer. After incubation on ice for 10 min, the cells were homogenized with an ice-cold Dounce tissue grinder. The homogenized mixture was centrifuged at 700 × g for 10 min at 4 °C. The supernatant was decanted into a new 1.5 ml microcentrifuge tube and centrifuged at 10,000 × g for 30 min at 4 °C. The pellet was resuspended in 1 × Cytosol Extraction Buffer and centrifuged again at 10,000 × g for 30 min at 4 °C, after which the supernatant was discarded. The pellet was resuspended in Enzyme Mix (5 μl) and incubated in a 50 °C for at least 60 min. The mixture was then centrifuged at 12500 × g for 5 min. The supernatant was discarded and the pellet (mtDNA) was resuspended in Tris–EDTA (TE) buffer. The mtDNA was then diluted to a concentration of 1 μg/μl for the subsequent experiments. A solution (0.02 μg/μl mtDNA) containing 1 μl of mtDNA (1 μg/μl), 47.5 μl of PBS, and 1.5 μl of Attractene Transfection Reagent (Qiagen, 301,005) was prepared and incubated at room temperature for 15 min before intravitreal injection. The control solution contained 48.5 μl of PBS and 1.5 μl of Attractene Transfection Reagent (Qiagen, 301,005). According to our previous study[14] in retinal microvascular endothelial cells stimulated with mtDNA, the concentration of mtDNA solution of intravitreal injection was 0.02 μg/μl. The rats were anesthetized and injected intravitreally with the mtDNA solution (0.02 μg/μl, 2 μl) or control solution (2 μl) 1 mm posterior to the limbus. After stimulation with mtDNA or control solution, the rats were maintained under their original feeding conditions. For electroretinography (ERG), the right eye of each rat was injected intravitreally with mtDNA and the left eye was injected with control solution. Only the right eye of each rat was included in the western blotting, TUNEL staining, and PCR analyses.

**ERG**
At 1, 3, 5, and 7 days after the intravitreal injection of mtDNA or control solution, retinal function was evaluated with ERG, recorded as a scotopic electroretinogram (Espion Electrophysiology System; Diagnosys LLC, Lowell, MA, USA). The rats were dark-adapted for 2 h and anesthetized. After the pupil was dilated with atropine sulfate (Santen Pharmaceuticals Co., Ltd), oxybuprocaine (Santen Pharmaceutical Co., Ltd) and carbomer (Bausch & Lomb, Rochester, NY, USA) were applied topically for corneal anesthesia and hydration, respectively. Under illumination with dim red light, platinum ring electrodes were placed on the corneal surface. A subdermal grounding electrode was placed hypodermically on the tail and an identical electrode inserted into the rat’s nose as the reference electrode. After 10 min dark adaptation, ERG signal recording was commenced as previously described [7]. The amplitude of the a-waves was measured from baseline to the troughs of the a-wave. The b-wave amplitude was measured from the negative peak of the a-wave to the positive peak of the b-wave.

**TUNEL Staining**

TUNEL staining was performed on the retinal sections using the In Situ Cell Death Detection Kit, TMR red (cat. no. 12156792910, Roche, Germany). The eyes were prepared and and removed the anterior segment of the eye 7 days after the intravitreal injection of mtDNA or control solution. 4% paraformaldehyde was used to fixed the eyecups, and then the eyecups were dehydrated in sucrose solutions (20% for 2 h and 30% for overnight). The tissues were embedded and stored at −80 °C and sagittal sections cut (10 μm). The tissue sections were fixed with 4% paraformaldehyde for 20 min at room temperature. The sections were immersed and washed in PBS for 30 min. The sagittal sections were then incubated with Permeabilization Solution (0.1% Triton X-100) for 30 min. The Enzyme Solution (vial 1) was added to the Label Solution (vial 2) to obtain the TUNEL reaction mixture (vial 1: vial 2 = 1:9). The sagittal sections were immersed in the TUNEL components and equilibrated for 60 min at 37 °C in a humidified atmosphere in the dark. After the sections were washed twice in PBS for 10 min each, 4¢,6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China) was used to counterstain the samples. The slides were observed and photographed under a fluorescence confocal microscope (Leica TCS SP8 WLL, Wetzlar, Hesse-Darmstadt, Germany). The approximate excitation/emission peaks of the TUNEL stain and DAPI were 364/454 and 540/580 nm, respectively.

**Western Blotting**

The retinas were collected at 1, 3, 5, and 7 days after the intravitreal injection of mtDNA or control solution. The retinas processed with the Mitochondria Isolation Kit for Tissue (C3606, Beyotime, Shanghai, China), as previously described[14] and the proteins collected from the supernatant and sediment were used for the detection of cytochrome c. Western blotting was performed according to methods described in previous study[14]. The following primary antibodies were used: rabbit anti-BAX (#2772, Cell Signaling Technology), rabbit anti-BAK (#12105, Cell Signaling Technology), rabbit anti-caspase 9 (AF6348, Affinity Biosciences Ltd), rabbit anti-cleaved caspase 9 (AF5240, affbiotech), rabbit anti-caspase 3 (ab44976, Abcam), rabbit anti-cleaved caspase 3 (ab49822, Abcam), anti-β-actin (ab8227, Abcam), anti-cGAS (ab179785, Abcam), rabbit anti-STING (D1V5L) (#50494, Cell Signaling Technology),
rabbit anti-TBK1/NAK (D1B4) (#3504, Cell Signaling Technology), rabbit anti-phospho-TBK1/NAK (Ser172) (D52C2) XP® (#5483, Cell Signaling Technology), rabbit anti-IRF3 (D83B9) (#4302, Cell Signaling Technology), rabbit anti-phospho-IRF3 (Ser396) (D601M) (#29047, Cell Signaling Technology), anti-interferon β (ab140211, Abcam), rabbit anti-cytochrome c (10993-1-AP, Proteintech), rabbit anti-VDAC1 (ab154856; Abcam). Three eyes from each group were tested.

**Real-time PCR**

The retinas were isolated 1, 3, 5, and 7 days after the intravitreal injection of mtDNA or control solution. The total RNA was isolated from each retina with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions, and quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Complementary DNA was synthesized with the PrimeScript RT Reagent Kit (Takara, Ohtsu, Shiga, Japan). A LightCycler® 480 II Real-Time PCR instrument (Roche, Basel, Switzerland) was used to perform all real-time PCRs as previously published study[14]. The primer sequences are shown in Table S1. Three eyes from each group were analyzed.

**Statistical Analysis**

Data were analyzed using the SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). The ERG results are the means of six rats were used per experiment. Differences between two groups were compared with the Mann–Whitney U test. The Kruskal–Wallis test was used to compare differences among three or more groups. Values of P < 0.05 were considered statistically significant. At least 3 independent experiments were conducted for the quantitative results.

**Results**

**Increased Cytosolic mtDNA Release in Retina after Injury**

The amount of cytosolic mtDNA in the retina, measured by PCR, was significantly increased at 3, 5, and 7 days after light injury (Figure 1A). The released mtDNA in the retina was highest after 3 days, when it was 2.5 times higher than in the normal control (Figure 1A). To provide further evidence that pathological stimuli cause the release of mtDNA, the cytosolic mtDNA was measured in rats after the intravitreal injection of LPS (100 ng/ml). As shown in Figure 1B, the amount of cytosolic mtDNA in the retina increased after LPS injection and was maximal after 7 days compared with the control level (Figure 1B).

**The cGAS–STING–TBK1–IRF3 Signaling Pathway is Activated by Cytosolic mtDNA**

After the retinal transfection of mtDNA, the transcription of cGAS mRNA increased significantly, as shown in Figure 2B. Cytosolic cGAS was then activated by the cytosolic mtDNA and was then degraded by P62-dependent ubiquitination, as previously reported[15]. Therefore, consistent with previous research, the amount cGAS protein decreased gradually after mtDNA stimulation (Figure 2A and 2C). STING, an endoplasmic reticulum (ER)-membrane protein, is activated by the cytosolic mtDNA–cGAS complex. After the retinal transfection of mtDNA, the transcription of Sting increased (Figure 2D), accompanied by an
increase in STING expression (Figure 2A and 2E). The phosphorylation of IRF3 and TBK1 was significantly increased by stimulation with mtDNA at 1, 3, 5, and 7 days after mtDNA stimulation (Figure 2A, 2F, and 2G). Simultaneously, mtDNA stimulation also promoted the transcription and expression of IFN-β in the retina at 1, 3, 5, and 7 days after transfection (Figure 2A, 2H, and 2I).

**Retinal mtDNA Stimulation Induces Retinal Apoptosis**

A TUNEL assay was used to assess the retinal cell damage at 7 days after the retinal transfection of mtDNA. As shown in Figure 3, compared with the normal control, the number of TUNEL-positive nuclei was significantly increased in the transfected retinas, and they were located in the ganglion cell complex (GCC), inner retinal nuclear layer (INL), and outer retinal nuclear layer (ONL) (control group, 3 ± 1 TUNEL-positive cells; mtDNA-transfected group, 132.67 ± 11.24 TUNEL-positive cells; these are the total numbers of TUNEL-positive cells, P < 0.01). Consistent with the TUNEL staining results, the caspase 9/caspase3 signaling pathway was activated by mtDNA stimulation. Caspase 9 was activated and increased time-dependently on days 3, 5, and 7 after stimulation (Figure 4A–C). Cleaved caspase 3 was also notably elevated 1 day after the intravitreal injection of mtDNA and peaked at 7 days. We then investigated the effects of caspase 9 and caspase 3 signal activation. The transcription and expression of proapoptotic proteins BCL-2-associated X (BAX) and BCL-2 antagonist/killer 1 (BAK) was significantly increased (Figure 4A and 4D–G) at 1, 3, 5, and 7 days after retinal transfection with mtDNA, accompanied by the increasing release of cytochrome c from the mitochondria to the cytosol (Figure 4A and 4H–I).

**Retinal mtDNA Stimulation Causes Retinal Dysfunction**

ERG was used to evaluate retinal function after mtDNA stimulation. The representative images were recorded sequentially (Figure 5). No significant differences were detected in the a-wave amplitude on rod-ERG of the mtDNA-transfected group and the normal control group (Figure 6A). The amplitudes of the a-waves on max-ERG were significantly lower in the eyes after mtDNA stimulation than in the normal control eyes at 1 day (−119.7 ± 45.1 vs −69.6 ± 40.2 μV, respectively; P < 0.05), 3 days (−117.6 ± 43.5 vs −65.1 ± 53.2 μV, respectively; P < 0.05), and 7 days after mtDNA stimulation (−101.2 ± 37.7 vs −76.1 ± 40.0 μV, respectively; P < 0.05) (Figure 6B). Figure 6C shows that there were no significant differences in the a-wave amplitudes on cone-ERG between the mtDNA eyes and control eyes. The amplitudes of the b-waves on rod-ERG in the mtDNA-transfected rats were 128.1 ± 74.7 μV, 102.3 ± 97.3 μV, and 92.5 ± 48.0 μV on days 1, 3, and 7, respectively, whereas in the control eyes, the b-wave amplitudes were 185.7 ± 74.8 μV, 181.4 ± 99.4 μV, and 181.7 ± 91.3 μV, respectively (Figure 6D) (all P < 0.05). Like the b-waves on rod-ERG, the amplitudes of the b-waves on max-ERG were significantly lower in the mtDNA-transfected eyes than in the control eyes, at 187.1 ± 91.6 μV, 131.1 ± 127.1 μV, and 143.5 ± 107.1 μV on 1, 3 and 7 days after the intravitreal injection of mtDNA, respectively (all P < 0.05; Figure 6E). The b-wave amplitudes on cone-ERG (Figure 6F) were also lower than in the control eyes on 1 day (52.3 ± 27.6 μV vs 63.1 ± 20.3 μV, respectively; P < 0.05) and 3 days after mtDNA stimulation (36.1 ± 21.5 μV vs 68.6 ± 28.5 μV, respectively; P < 0.05). Figure 6G shows that on flicker-ERG, the b-wave amplitudes were also reduced by mtDNA at 3
days relative to the control values (26.9 ± 16.2 μV vs 54.5 ± 19.4 μV, respectively; P < 0.05). Therefore, mtDNA stimulation induces retinal dysfunction in rats.

Discussion

Retinal degeneration occurs in a group of common retinal diseases that involve the progressive deterioration of retinal photoreceptor cells, eventually culminating in their death, which can lead to visual impairment[16]. These diseases include light-induced retinal degeneration[17], age-related macular degeneration[18], and inherited retinal degeneration[19]. Despite recent advances in our understanding of the mechanisms underlying these retinal degenerative diseases, their molecular pathology remains unresolved, especially for age-related macular degeneration and retinitis pigmentosa[19, 20]. Much work is still required to clarify the exact mechanism of each disease. In the present study, we have demonstrated that mtDNA escapes from the mitochondria into the cytosol after retinal injury. We isolated this mtDNA and used it to transfect the retinas of rats. We found that cytosolic mtDNA is recognized by the DNA sensor protein cGAS, which triggers the activation of the STING–TBKI–IRF3–IFN-β signaling pathway. The increased secretion of IFN-β leads to the activation of noncanonical inflammation and promotes retinal injury. We also found that cytosolic mtDNA triggers the activation of the proapoptotic effector proteins BAX and BAK. After the activation of BAX and BAK, these proteins accumulate at the mitochondrial outer membrane and induce its permeabilization, causing the release of cytochrome c into the cytoplasm. This cytosolic cytochrome c then activates caspase 3 and caspase 9, resulting in apoptosis.

The cells of the retinal neuroepithelial layer are terminally differentiated neurons, with no regenerative ability. Injuries such as infection, ischemia, and trauma in retinal neuroepithelial layer cells can lead to visual impairment and even blindness[21]. Some retinal degenerative diseases involve progressive photoreceptor cell loss and the degradation of RPE cells[22]. These diseases significantly affect the quality of life of millions of people each year. Previous studies have shown that degenerative diseases are the commonest cause of vision loss in those over the age of 65 in developed countries[23]. Furthermore, their prevalence is likely to increase with the exponential ageing of the population[24]. The projected number of people with age-related macular degeneration in 2040 is 288 million[25]. Many studies have focused on understanding retinal cell damage, and inflammation and apoptosis are recognized as playing central roles in these progressive diseases[26, 27].

Inflammation is a plausible link between mitochondrial damage and retinal disease. Research has demonstrated that the activation of retinal inflammation induces cell damage, such as the loss of retinal ganglion cells in glaucoma[28], the apoptosis of pigment epithelium cells in retinitis pigmentosa[26], the dysfunction of retinal photoreceptor cells in age-related macular degeneration[21], and even retinal detachment[29], which can progress and induce an inflammatory response, resulting in retinal atrophy. After cellular stress or tissue injury, cells release intracellular molecules into the extracellular space, which trigger the stress signals called DAMPs. DAMP molecules are regarded as endogenous danger signals because they induce potent inflammatory responses by activating the innate immune system during
noninfectious inflammation[30]. The mitochondrial DAMPs have been identified as important mediators of the innate immune response and are implicated in various degenerative conditions[31]. The mitochondria are evolutionary endosymbionts that were derived from bacteria[32], with which they share numerous similarities, including significant amounts of unmethylated CpG motifs in their genomes, which also encode essential protein subunits of the oxidative phosphorylation system[33]. Recent studies have shown that mtDNA is recognized by some important pattern recognition receptors (PRRs) of the innate immune system, TLR9, cytosolic inamasomes, and type I interferon[34–36]. mtDNA is also recognized by another DNA sensing pathway, in which cGAS interacts with mtDNA and its receptor to trigger a proinflammatory response[12]. CGAS, a cytosolic DNA sensor, activates the innate immune response in various cells. Some research have demonstrated that the mtDNA can be recognized and bound to cGAS with intermolecular hydrogen bonds[37]. Then the binary cGAS-DNA complex promoted the generation of cGAMP from GTP and ATP[38]. Nagaraj Kerur et al. demonstrated that RPE degeneration in human cell culture and in mouse models is driven by a noncanonical inamasome pathway[12]. In that study, they noted that when mtDNA escapes into the cytosol, it engages cGAS, causing the elevation of caspase 4, gasdermin D, and IFN-β levels in the RPE. This suggests that cGAS-driven IFN signaling is a conduit for mitochondrial-damage-induced inamasome activation in noninfectious human diseases, such as age-related macular degeneration.

Our previous studies have demonstrated light-injury and intravitreal LPS injection can lead to the inflammatory response or apoptosis in retina in vivo[5–8]. We also found that pathological stimulation can induce mtDNA leak into the cytosol of retinal microvascular endothelial cells. The DNA sensor cGAS was activated by the escaped mtDNA, with the subsequent expression of inflammatory cytokines[14]. The findings of the present study suggest that light- or LPS-induced injury also leads to the release of mtDNA in the retinal tissue in vivo. The cGAS–STING–TBK1–IRF3 signaling pathway was activated and IFN-β accumulated after transfecting the retina with cytosolic mtDNA. Therefore, we speculate that stimulation, such as light-injury and intravitreally injected LPS, causes the retinal neuroepithelial layer cells to release mtDNA into their cytoplasm, which then activates the cGAS–STING signaling axis, mediating the inflammatory response and retinal damage.

Apoptosis is a noninflammatory form of cell death that has been identified in many diseases[39]. It quickly and efficiently removes dead cells without inducing an immune response. It is involved in retinal neuroepithelial dysfunction in a variety of retinal diseases, including retinitis pigmentosa, age-related macular degeneration, and glaucoma[17, 28, 40]. We have shown that retinal neuroepithelial cells, including retinal ganglion cells, bipolar cells, and photoreceptor cells, underwent apoptosis after the transfection of the retina with cytosolic mtDNA, and that caspase 3 and caspase 9 were strongly activated. When we investigated this activation of caspase 3 and caspase 9, we found that the transfection of mtDNA induced the transcription and translation of the proapoptotic molecules BAX and BAK, members of the BCL-2 family, which controls and regulates the intrinsic (or mitochondrial) apoptotic pathway. Members of the BCL-2 family are classified into three groups according to their function in apoptosis and the number of BCL-2 homology (BH) domains they contain[41]. The proapoptotic effector proteins BAX and BAK accumulate and induce mitochondrial outer membrane permeabilization, leading
to the release of cytochrome c[42]. The maintenance of cytochrome c inside the mitochondrion is essential for the mitochondrial respiratory function[43]. Once released into the cytosol, cytochrome c binds to apoptotic peptidase activating factor 1 (APAF1), activating the formation of the ring-like apoptosome[44, 45]. Apoptosomes activate procaspase 9, which in turn activates the effector caspase 3, resulting in the activation of the apoptotic caspases[46, 47]. However, it is still unclear how cytosolic mtDNA promotes the upregulation of the proapoptotic molecules BAX and BAK. Researchers have shown that the BH3 domain of IRF3 interacts directly with BAX, and thus activates BAX through conformational changes[48–50]. Therefore, we infer that retinal transfection with mtDNA induces cell apoptosis through the cGAS–STING–TBK1–IRF3 axis[51]. How the complex regulation of apoptosis by mitochondrial damage and the release of mtDNA into the cytoplasm trigger apoptosis must be examined in detail in future research.

These types of damage all lead to retinal dysfunction, either through the retinal inflammatory response or retinal neuronal apoptosis. We used ERG to evaluate the effects of cytosolic mtDNA on retinal function in vivo and detected significant attenuation of the wave amplitudes on ERG. We conclude that stimulation by mtDNA causes significant retinal dysfunctions in vivo.

**Conclusion**

Pathological stimulation induces the release of mtDNA into the cytosol of retinal cells[12, 14]. This cytosolic mtDNA is recognized by the DNA sensor protein cGAS and triggers the cGAS–STING–TBK1–IRF3–IFN-β pathway, leading to the accumulation of IFN-β and the activation of the inflammatory response. Cytosolic mtDNA also promotes the expression of the proapoptotic molecules BAX and BAK, leading to the release of cytochrome c and the subsequent activation of caspases 3 and 9, causing mitochondrion-induced apoptosis and retinal dysfunction in vivo. In the next study, we will further explore whether the prevention of mtDNA release can inhibit the inflammation and apoptosis in retina.

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the Ethics Committee of the Eye and Ear Nose Throat Hospital of Fudan University, Shanghai, China.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Not applicable.

**Competing interests**
None of the authors of this manuscript has any conflict of interest to declare.

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**Author contributions**

YG performed the study and wrote the paper; RG designed and performed the study; DG, FH, YC, JY, BL, and QS performed the data analysis; GX revised the paper.

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Figures
Figure 1

Real-time PCR was used to measure the copy numbers of mtDNA in the cytoplasm of retinal tissue. Copies of mtDNA released from the mitochondria into the cytoplasm of retinal tissues of the control and after light-exposure at 1, 3, 5, and 7 days (A) or control and an intravitreal injection of LPS (2 μl, 100 ng/ml) (B) at 1, 3, 5, and 7 days. *P < 0.05, **P < 0.01, n = 3 biological replicates in each group.
Figure 2

A: Western blotting was used to evaluate the changes in proteins in retinas at 1, 3, 5, and 7 days after mtDNA stimulation (0.02 μg/μl, 2 μl). β-Actin was used as the loading control. Real-time PCR was used to evaluate the transcription of cGAS (B), STING (D), and IFNB1 (I) in retinas after stimulation with mtDNA (0.02 μg/μl, 2 μl) for different times. Western blotting analysis of cGAS (C), STING (E), phospho-TBK1 (F), phospho-IRF3 (G), and INF-β (H) in retinas after stimulation with mtDNA (0.02 μg/μl, 2 μl) for different times. *P < 0.05, **P < 0.01, n = 3 biological replicates in each group.

Figure 3

mtDNA stimulation (0.02 μg/μl, 2 μl) caused retinal cell damage, assayed with TUNEL staining after 7 days. (A) Representative retinal sections from the mtDNA retinal transfection group and normal control
group. GCC, ganglion cell complex; INL, inner retinal nuclear layer; ONL, outer retinal nuclear layer. (B) Statistical analysis of the percentage of TUNEL-positive cells in each group. In each section, two areas 500 µm away from the optic nerve head, were selected for analysis. The number of TUNEL-positive cells was counted in each visual field and averaged. Only one section was chosen from each eye. n = 3 biological replicates in each group; **P < 0.01, scale bar: 50 µm.

Figure 4

Mitochondrial apoptotic signaling pathway was activated by mtDNA stimulation (0.02 µg/µl, 2 µl). (A) Western blotting of retinal BAK, BAX, cleaved caspase 9, cleaved caspase 3, and cytochrome c protein levels in the normal control group and mtDNA-transfected groups after 1, 3, 5, and 7 days. Western blotting analysis of cleaved caspase 3 (B), cleaved caspase 9 (C), BAK (F), and BAX (G) in retinas after stimulation with mtDNA (0.02 µg/µl, 2 µl) for different times. Real-time PCR was used to evaluate the transcription of Bax (D) and Bak (E) in retinas after stimulation with mtDNA (0.02 µg/µl, 2 µl) for different times. Western blotting of cytochrome c expression in cytosolic (H) and mitochondrial extracts (I) of retinas from each group. β-Actin was used as the loading control. VDAC1 was used as the internal control for mitochondrial proteins. *P < 0.05, **P < 0.01, n = 3 biological replicates in each group.
Figure 5

Retinal function at 1, 3, and 7 days after mtDNA stimulation. Representative images of α- and β-waves on rod-ERG, max-ERG, cone-ERG, and flicker-ERG in the control (left) eyes and mtDNA-treated (right) eyes were recorded, in that order, using the Espion Visual Electrophysiology system.
Figure 6

Statistical analysis of the amplitudes ($\mu$V) of the a- and b-waves on ERG at 1, 3 and 7 days after mtDNA stimulation (0.02 $\mu$g/$\mu$l, 2 $\mu$l). The right eye of each rat was intravitreally injected with mtDNA and the left eye of each rat was intravitreally injected control solution. (A) The a-wave amplitudes on rod-ERG. (B) The a-wave amplitudes on max-ERG. (C) The a-wave amplitudes on cone-ERG. (D) The b-wave amplitudes on rod-ERG. (E) The b-wave amplitudes on max-ERG. (F) The b-wave amplitudes on cone-ERG. (G) Flicker-ERG amplitudes. Data are means ± SD, n = 6 per group, *P < 0.05.

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