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Leber's Hereditary Optic Neuropathy (LHON): Optimisation of *MT-ND4* Cell Model

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ABSTRACT Leber's Hereditary Optic Neuropathy (LHON) is a maternally inherited optic neuropathy that leads to vision loss. Three primary mitochondrial DNA mutations (*MT-ND1*, *MT-ND4* and *MT-ND6*) are the main cause of the disease, which affects the function of respiratory complex 1 of oxidative phosphorylation. As yet there is no proper treatment for this disease and many studies are going on to find a solution for this condition. In this paper, the researchers discussed the basic cell culture method for LHON ND4 mutant fibroblast cell maintenance for basic research. Cell viability and morphology analysis followed by proper cell maintenance using appropriate media and conditions helped to attain the confluency of cells in each passage. Confluent cells were cryopreserved for long-term storage for further experiments and assays.

INTRODUCTION

Human fibroblasts are the principal active cells derived from connective tissue with irregular and elongated spindle morphology. These adherent cells produce a large amount of extracellular matrix (ECM) proteins such as collagen, glycosaminoglycans and proteoglycans (Villegas et al. 2005). Fibroblasts' ease of use, availability and robustness makes the skin fibroblasts an important *in vitro* model for various studies. It has been used for both primary cultures and for generating permanently transformed cell lines. Human fibroblast cells have been used as a model to explore the pathophysiology mechanisms of several diseases (Ambrosi et al. 2014; Hu et al. 2015) due to their replication of cumulative cell damage, mitochondrial DNA polymorphisms and mutations (Auburger et al. 2012). All these characteristics of these *in vitro* models will make these cell lines a valid tool for basic and translational research development.

Leber's Hereditary Optic Neuropathy (LHON) is an inherited mitochondrial disorder associated with mitochondrial point mutations m.3460G>A in *ND1*, m.11778G>A in *ND4*, and m.14484T>C in *ND6* (7), which optic neuropathy and that leads to vision loss. LHON initiates subacute unilateral visual acuity loss, dyschromatopsia in the red-green axis and a centrocecal scotoma. Another eye develops symptoms within a few months of the onset of diseases but in 25 percent of cases, diseases begin bilaterally. Most of the patients persist with visual acuity of less than 0.1, however, a small percentage of patients experience a natural slight progress in visual acuity (Priglinger et al. 2019). These genes code for the subunit of nicotinamide adenine dinucleotide: ubiquinone oxidoreductase (complex I) of the mitochondrial respiratory chain. These mutations in complex 1 eventually disrupt the oxidative phosphorylation mechanism and cause an imbalance in ATP and reactive oxygen species (ROS) production in a cell. Alterations in mitochondrial functioning affect cell viability and promote cell death (Yu-Wai-Man et al. 2011; Devi et al. 2021a). The mechanism behind the optic nerve cell death and specific features of LHON condition due to these genetic changes are still unclear. Studies have reported that LHON patients with the primary mutation have a drastic reduction in the complex1 activity of mitochondria (Devi et al. 2021b; Carelli et al. 2004). Currently, there is no proper treatment option for LHON other than some supportive medicines. Gene therapy studies have been considered as an option for restoring the activity of complex 1 and it is still under clinical trials. Other than gene therapy, experimental studies have

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been reported to reduce disease progression and restoration of vision (Bahr et al. 2020). Inadequacy of a proper model system for LHON and lack of understanding of the pathophysiology of the disease is one of the significant problems in developing appropriate treatment (Bahr et al. 2020).

Cell models such as peripheral blood from LHON patients, cybrids, lymphoblast and fibroblast are being used for the experimental purposes of understanding electron transport chain shortfall especially related to complex 1 activity and pathogenesis of LHON (Angebault et al. 2011; Pecina et al. 2014). Fibroblast cell models are used in protein expression studies, ATP production and various electron transport chain complex functions. While considering all these cellular properties together, the prevailing fibroblast cell lines are an important tool for LHON research. The recent study aims to optimise the LHON mutant fibroblast cells for the laboratory conditions to conduct further experiments with various treatments and validation studies.

MATERIAL AND METHODS

LHON ND4 Mutant Fibroblast Cell Line Culture

Skin derived LHON ND4 mutant fibroblast cells were gifted by Dr. Patrick Yu Wai Man's lab, University of Cambridge, UK. The cells were cultured in 4.5g/ml high glucose DMEM (Sigma) with 10 percent Foetal Bovine Serum (FBS) supplemented with 1 percent of L-glutamine (Gibco), 1 percent of non-essential amino acid (NAA) (Sigma), 100U/ml penicillin, and 100µg/ml streptomycin (Sigma). The culture flask was incubated in humidified 5 percent CO_2 at 37^0C. Media were changed every 2 days and the cells were passaged when the cell confluency reached approximately 70-80 percent for cell line maintenance. For the subculturing, after aspirating the culture medium, the culture flask is rinsed with PBS twice and then added with 1 ml of 0.25 percent trypsin with EDTA and maintained in an incubator for five minutes. Then the trypsinized cells were collected and centrifuged at 1200 rpm for 5 minutes. Collect the pellet and re-seed the cells in 1×10^4 cells per cm^2.

Morphological Analysis of Cells

Cells were seeded in T25 flasks at an initial seeding density of 2×10^5 cells and incubated overnight at 37^oC in 5 percent CO_2. After 24 hours, the morphology of fibroblast cells was observed by phase contrast microscopy using Primovert digital microscope, Zeiss equipped with a camera, Axiocam 208 colour (Carl Zeiss, Germany). Images were taken in different magnifications and morphology was assessed each day. Cells were passaged when they achieved 80 percent growth coverage of the total surface area.

Trypan Blue Cell Viability and Cell Counting Assay

Cell number and cell viability (live, dead and total cells) were measured using the trypan blue exclusion technique. Cell counts were determined using a haemocytometer. For the assay, culture media were aspirated and washed the cells with 1mL of PBS. Trypsinization of cells was done with 0.025 percent trypsin by incubating for 2-5 minutes. Once cells were detached, they were resuspended with pre-warmed (37^0C) media and centrifuge at 1200 rpm for 3 minutes and pellets were resuspended in fresh medium. Cell counting was performed by mixing 10µL of sample mixing with 10µL of 0.4 percent trypan blue (1:1 ratio). 10µL of the cell-trypan blue mix was pipetted into the glass slide, which was placed in the Neubauer haemocytometer. Viable cell counting was performed under a phase-contrast microscope in a clear field (Primovert, Zeiss).

DNA Extraction and Genotyping of Cells

LHON ND4 mutant cells were grown in T75 cell culture flasks until 80 percent confluency. Then the cells were trypsinised and counted in the Neubauer Haemocytometer by adding a trypan blue stain. Approximately 5×10^6 cells were taken for the DNA isolation using QIAamp® DNA Mini Kit (QIAGEN) following the standard protocol. DNA was quantified using a Nanodrop (Spectrophotometer ND-1000). After DNA isolation, the *MT-ND4* gene was amplified using specific primers (Forward primer-5'GCT CCC TTC CCC TAC TCA TC 3': Reverse primer 5'AGG GGT CGT AAG CCT CTG TT 3') and amplification is confirmed using 2 percent agarose gel electrophoresis and proceeded for DNA sequencing. Both 3' to 5' strand and 5' to 3' strand of the amplicon was subjected to cycle sequencing by the BigDye Terminator cycle se-

quencing kit (Applied Biosystems, CA). Then DNA was precipitated by adding Ethanol and 5M sodium acetate in a 5:1 ratio. Then the sequences were analysed using ABI 3500 Genetic Analyser (Applied Biosystems, Foster City, CA) after being dissolved in Hi-Di formamide to reduce high-quality sequencing. The *MT-ND4* sequence was analysed using BioEdit Sequence Analyser software to identify the target mutation.

Restriction Digestion for Zygosity Analysis

The zygosity of the ND4 mutation in the cell lines was determined by restriction digestion of the *MT-ND4* gene. The *MT-ND4* gene was amplified by PCR and then subjected to restriction digestion. The enzyme NmuCI (Tsp45I) was used to digest the restriction site in the ND4. In 5µl of the PCR product, 0.2µl of NmuCI (Tsp45I) enzyme with 2 µl of buffer was added and incubated at 37^0C for 16 hours to ensure complete digestion of the *MT-ND4*. The digested product was then run in 4 percent agarose gel (Seakem® LE Agarose, Lonza, Rockland, ME) to visualise the size of the fragments.

Cryopreservation of Cells

Detached fibroblast cells were counted in a Neubauer chamber and 1×10^6 viable cells were directly transferred into cryovials and resuspended in 1 mL chilled cryopreservation medium containing 50 percent above mentioned complete medium appropriate for the cell type, 40 percent FBS (Gibco) and 10 percent DMSO (Sigma). Cryovials were immediately transferred to an isopropyl alcohol-based controlled rate freezing container and placed in a –80°C freezer. After 24 hours, vials were transferred to liquid nitrogen at -196^0 C for cryogenic storage where they can be maintained for the long term.

RESULTS

Cell Culture Maintenance

The researchers have established and maintained LHON ND4 mutated fibroblast cell culture using appropriate media and supplements for the growth at ideal pH conditions. This pH maintenance will balance cellular proliferation and biosynthesis activity of fibroblast cells. Cells obtained 80 percent confluency within 8-9 days (Fig. 1) and the subculture of cells was done with 80 percent confluency of cells. In subculturing, trypsinised cells were dissociated from the flask with spherical morphology as smaller cell clumps and single cells. These cells reseeded into new flasks and cell morphology, cell number and cell viability were evaluated at every passage. The researchers were able to achieve a reliable culture of LHON ND4 mutant fibroblast cells displaying uniform cellular morphology, higher viability, proliferation potential and genome stability throughout the culture period.

Morphological Assessment of Cells

Cellular morphology was routinely examined using phase contrast microscopy. Morphological analysis of LHON fibroblast cells revealed that cells were round in the initial stage of adhesion. More

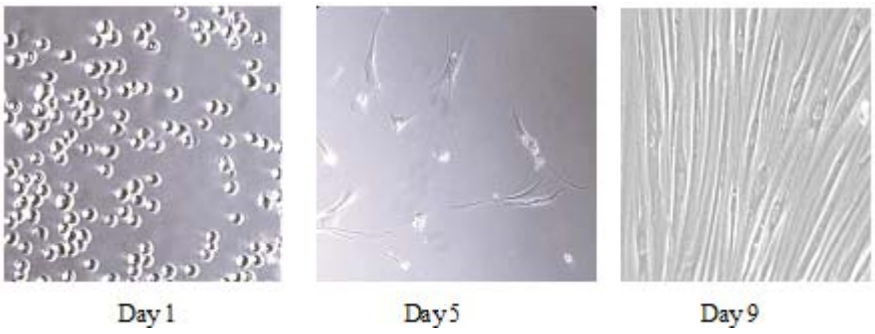

Fig. 1. Examination of attachment and growth of fibroblast cells at various periods of growth. Images of cultured cells were captured on day 1, day 3 and day 9

number of cells attached to the flask surface at 48-56 hours and most of the cells were attached and initiated spreading at 72 hours. Within 8-9 days cells were able to form a compact monolayer with 80-90 percent confluency (Fig. 2). Fibroblast cells appear elongated, flat and spindle-shaped with a centrally placed oval or round-shaped nucleus.

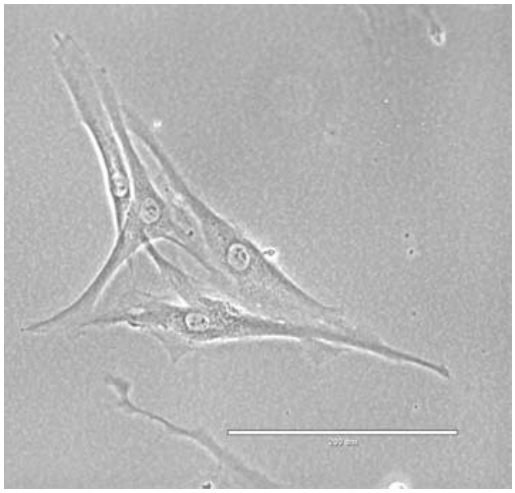

Fig. 2. Morphology of LHON ND4 mutant cell is analysed using a phase contrast microscope. Cells displayed exact fibroblast cell morphology with flat and spindle shape

Trypan Blue Exclusion Assay

Trypan blue assay estimated the total cell number and viability of cells. Trypan blue is a membrane-impermeable dye, which live cells could not take up. In dead cells, the dye is penetrable inside the cell through a disoriented plasma membrane and the cell could be visible in blue colour. This dye permeability changed the colour of the cell and it helps to distinguish viable cells from dead cells. Examination of cells using microscopy is the most widely established technique to assess the live cells and cell count using trypan blue assay.

Genotyping and Mutational Confirmation of Cells

LHON *ND4* mutation status was determined by sequence analysis of the mitochondrial genome using Sanger sequencing from the fibroblast cell lines (Fig.3). PCR-based restriction digestion was performed to determine the plasmic status of the mutations (Fig.4). In this study digestion of PCR products was conducted with Tsp45I revealed homoplasmic ND4 mutation condition.

DISCUSSION

Mitochondrial ND4 (m.11778G>A) mutation causes the majority of Leber's Hereditary Optic

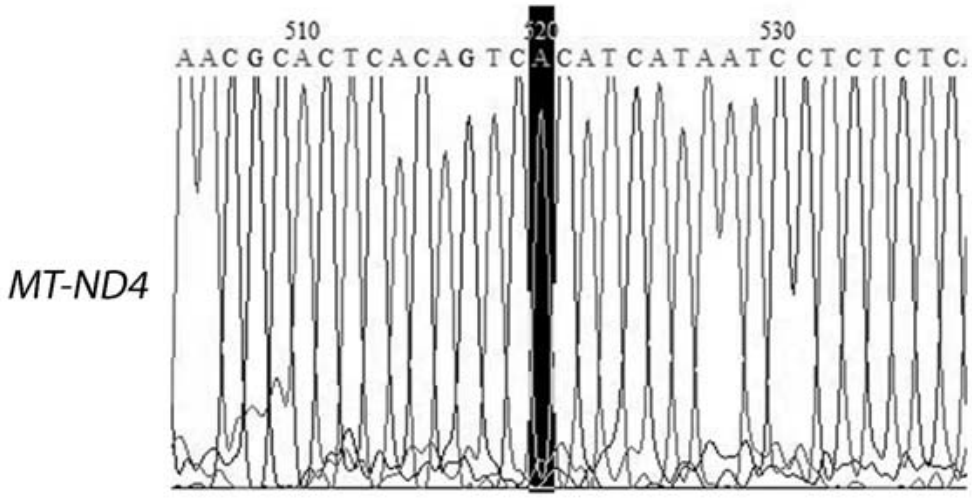

Fig. 3. Electropherogram and PCR-RE LHON ND4 m.11778G>A mutation

Neuropathy (LHON) cases worldwide (Yu-Wai-Man et al. 2011). Oxidative stress and ROS generation are significantly associated with mitochondrial dysfunction, which is evaluated in various retinal and ocular diseases (Subramaniam et al. 2020). Since the pathogenic mechanism of LHON is still unclear, treatment options are limited and sustained research is required for offering effective and safe treatment for patients. Many promising novel treatment options are presently being evaluated including gene therapy options and several clinical trials are ongoing in recent potential treatments for LHON (Subramaniam et al. 2022). Gene therapy techniques include allotopic expression, which allows mitochondrial protein expression through incorporating new genetic material into nuclear DNA that can be targeted to the mitochondria (Koilkonda et al. 2014; Cwerman-Thibault et al. 2015). For better therapeutic options for patients, basic and clinical research should be integrated to make an effective transition from bench side to bedside.

Fibroblast cells can be maintained in the active multiplication stage for a long time. Robustness of the cell and minimal doubling time makes the *in vitro* expansion of fibroblast cells easier. These characteristics of fibroblast cells mould it as an ideal tool for studying diseases from patient-derived skin fibroblast cells *in-vitro* (Fernandes et al. 2016). Fibroblast cells-based studies helped to clarify the pathogenesis of various diseases in medical research (Hu et al. 2015; Tun et al. 2014). In this study, LHON *ND4* mutant fibroblast was used and this mutation has adverse effects on the functioning of mitochondrial complex 1. This impairment in complex 1 function would diminish the cellular metabolism and growth. Here the attachment and cells to reach confluency require more time period than the normal cells after thawing. At the cell confluency stage, cells were trypsinised and cryopreserved in liquid nitrogen for long-term storage. Morphological features were analysed regularly to identify the cellular shape and cellular regularity using a phase contrast microscope at various magnifications. *ND4* mutation was confirmed by Sanger sequencing and restriction enzyme digestion analysis provided plasmic status of the mutation. These maintained LHON mutant cells were cryopreserved and stored for further experiments and assays. The study highlights the importance and basic *in vitro* cell culture condition for LHON *ND4* mutant cell proliferation.

CONCLUSION

The results demonstrated the successful culturing of LHON mutant cells using defined media and other conditions. Better-quality cell growth was observed and cells conquered 80-90 percent confluent by 5-6 days with fibroblast cellular morphology. This establishment of cultural conditions will facilitate the conduct of further functional studies and assays using these cell lines.

RECOMMENDATIONS

It is essential to develop a therapeutic approach for Leber's Hereditary Optic Neuropathy (LHON) since there are no treatment options available. Cellular studies are the preliminary steps of translational research and they would help to develop a therapeutic solution from bench to bedside. Further studies using these LHON mutant cells can ensure translational research for LHON therapeutics.

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REFERENCES

Ambrosi G, Ghezzi C, Sepe S, Milanese C, Payan-Gomez C et al. 2014. Bioenergetic and proteolytic defects in fibroblasts from patients with sporadic Parkinson's disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1842: 1385-1394.

Angebault C, Gueguen N, Desquiret-Dumas V, Chevrollier A et al. 2011. Idebenone increases mitochondrial complex I activity in fibroblasts from LHON patients while producing contradictory effects on respiration. *BMC Research Notes*, 4: 557.

Auburger G, Klinkenberg M, Drost J, Marcus K, Morales-Gordo B et al. 2012. Primary skin fibroblasts as a model of Parkinson's disease. *Molecular Neurobiology*, 46: 20-27.

Bahr T, Welburn K, Donnelly J, Bai Y 2020. Emerging model systems and treatment approaches for Leber's hereditary optic neuropathy: Challenges and opportunities. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1866: 165743.

Carelli V, Ross-Cisneros FN, Sadun AA 2004. Mitochondrial dysfunction as a cause of optic neuropathies. *Progress in Retinal and Eye Research*, 23: 53-89.

Cwerman-Thibault H, Augustin S, Lechauve C, Ayache J, Ellouze S et al. 2015. Nuclear expression of mitochondrial ND4 leads to the protein assembling in complex I and prevents optic atrophy and visual loss. *Molecular Therapy-Methods & Clinical Development*, 2: 15003.

Devi SM, Kumar BA, Mahalaxmi I, Balachandar V 2021b. Leber's hereditary optic neuropathy: Current approaches and future perspectives on mesenchymal stem cell-mediated rescue. *Mitochondrion*, 60: 201-218.

Devi SM, Nair AP, Mahalaxmi I, Balachandar V 2021a. Mitochondrial function and epigenetic outlook in Leber's Hereditary Optic Neuropathy (LHON). *Neurology Perspectives*, 4: 220-232.

Fernandes IR, Russo FB, Pignatari GC, Evangelinellis MM, Tavolari S et al. 2016. Fibroblast sources: Where can we get them? *Cytotechnology*, 68: 223-228.

Hu W, Qiu B, Guan W, Wang Q, Wang M et al. 2015. Direct conversion of normal and Alzheimer's disease human fibroblasts into neuronal cells by small molecules. *Cell Stem Cell*, 17: 204-212.

Koilkonda RD, Yu H, Chou TH, Feuer WJ, Ruggeri M et al. 2014. Safety and effects of the vector for the Leber hereditary optic neuropathy gene therapy clinical trial. *JAMA Ophthalmology*, 132: 409–420.

Pecina P, Houš•ková H, Mráèek T, Pecinová A, Nùsková H et al. 2014. Noninvasive diagnostics of mitochondrial disorders in isolated lymphocytes with high resolution respirometry. *BBA Clinical*, 2: 62-71.

Priglinger C, Klopstock T, Rudolph G, Priglinger SG 2019. Leber'sche hereditäre Optikusneuropathie. (Leber's Hereditare Optic Neuropathy) *Klin Monatsblätter für Augenheilkunde*, 236: 1271-1282.

Subramaniam MD, Chirayath RB, Iyer M, Nair AP, Vellingiri B 2022. Mesenchymal stem cells (MSCs) in Leber's hereditary optic neuropathy (LHON): A potential therapeutic approach for future. *International Ophthalmology*, 1-16.

Subramaniam MD, Iyer M, Nair AP, Venkatesan D, Mathavan S et al. 2020. Oxidative stress and mitochondrial transfer: A new dimension towards ocular diseases. *Genes & Diseases*, 9: 610-637.

Tun AW, Chaiyarit S, Kaewsutthi S, Katanyoo W, Chuenkongkaew W et al. 2014. Profiling the mitochondrial proteome of Leber's hereditary optic neuropathy (LHON) in Thailand: Down-regulation of bioenergetics and mitochondrial protein quality control pathways in fibroblasts with the 11778G> a mutation. *PLoS One*, 9e106779.

Villegas J, McPhaul M 2005. Establishment and culture of human skin fibroblasts. *Current Protocols in Molecular Biology*, 71: 28.3.1-28.3.9. https://doi.org/10.1002/0471142727.mb2803s71

Yu-Wai-Man P, Griffiths PG, Chinnery PF 2011. Mitochondrial optic neuropathies- disease mechanisms and therapeutic strategies. *Progress in Retinal and Eye Research*, 30: 81–114.

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