BF-1803
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RESEARCH ARTICLE Indian Journal of Animal Research

# Effects of Rotenone Exposure on Apoptosis in rAAV-*NDI1*-infected Neural Stem Cell Line of Minipig

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## ABSTRACT

**Background:** The overall objective of this study is to confirm that normal expression of yeast NADH- dehydrogenase (*NDI1*) can occur in neural stem cell lines of minipig with resistance to rotenone exposure, an environmental factor responsible for dysfunction of mitochondrial enzyme complex I. In modern society, there are many diseases that cannot be treated. Diseases including LHON, Parkinson disease and dystonia, have been associated with defects in mitochondrial complexes. This experiment was performed to demonstrate that it was not sensitive. The overall objective of this study is to confirm that normal expression of yeast NADH-dehydrogenase (*NDI1*) can occur in neural stem cell lines of minipig with resistance to rotenone exposure, an environmental factor responsible for dysfunction of mitochondrial enzyme complex I.

**Methods:** A Mini Pig Neural stem cell line (MPV) was used for transfection of the NDI gene, DMEM/F-12 culture medium and MPV was inoculated in a six-well plate (Corning, USA) at a concentration of $1\times10^5$ cells/3ml/well, with inoculation of MPV at 37°C for 24 hours, 5% $CO_2$, followed by incubation in an incubator with 95% humidity and attached. The plate containing MPV cells was treated with recombinant adeno-associated virus ndi1 (rAAV-ndi1) for transfection, with periodic replacement with a cell selection culture solution containing 10% FBS, 1% P/S and 0.2 μM rotenone. The experiment was performed for restoration of mitochondrial activity of thawed cells. RNA was extracted from MPV cells and transfection of MPV cells with the *NDI1* gene was performed using Trizol (Invitrogen, USA). Reverse transcription PCR (RT-PCR) and Western blot were performed to confirm normal expression of the *NDI1* gene in MPV cells transfected with the *NDI1* gene. Immunofluorescence was performed to determine the presence of the *NDI1* protein in the cell and the cell count was used for LUNA and the rate of cell death for rotenone was determined using the MTS [3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay.

**Result:** rAAV-*NDI1* was successfully introduced into MPV cells and the proliferation rates for the cells were compared with those of the transformed cells; after three days, the non-infected cells were killed and the infected cells proliferated. The results after differentiating the cell lines were similar to those of previously reported studies. Toxicity analysis on rotenone was also performed using the MTS assay and the rates of cell death over three days were compared; the results showed significantly lower levels of NDI1-transformed minipig neural stem cells compared with those of minipig. The present work will be a complementary contribution to the comprehensive study of the scorpion sting syndrome. These results were similar to those of previously reported studies. Previous studies have reported that oxidoreductive stress can be a cause of apoptosis. Therefore, conduct of additional studies for measurement of ROS and oxygen utilization to determine oxidative stress in cells will be necessary.

**Key words:** Apoptosis, Minipig neural stem cells, Mitochondria complex I, *NDI1* gene, Parkinson disease.

## INTRODUCTION

After Alzheimer's disease (alzheimer's disease), Parkinson's disease is known as a common degenerative neurological disease worldwide. An estimated 7-10 million people are affected by this disease. Parkinson's disease is a progressive neurodegenerative disease affecting the nigrostriatal pathway responsible for body movements caused by dopaminergic regression and proteinaceous aggregates of cells known as lewy bodies.

The pathogenic mechanism involved in Parkinson's disease is still unknown, however, results of biochemical analysis have demonstrated that Parkinson's disease can be caused by mitochondrial dysfunction and genetic and environmental factors can be a cause of mitochondrial dysfunction. These findings have been reported in several studies (Spillantini *et al*., 1997; Wooten, 1997; Sherer *et al*., 2002; Betarbet *et al*., 2003).

Mitochondrial dysfunction, first reported in 1962 in a young female with over-utilization, was caused by structurally abnormal mitochondria and abnormal oxidative

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**How to cite this article:** Chun, H.J., Lee, D.M., Kim, S.U., Lee, H.J. and Seo, B.B. (2024). Effects of Rotenone Exposure on Apoptosis in rAAV-*NDI1*-infected Neural Stem Cell Line of Minipig. Indian Journal of Animal Research. DOI: 10.18805/IJAR.BF-1803.

**Submitted:** 10-04-2024 **Accepted:** 25-06-2024 **Online:** 25-07-2024

phosphorylation, now known as Luft's disease, which is rare (Luft, 1994). Since then, structural and functional defects of complex I-V have been reported as a cause of mitochondrial dysfunction (Wallace, 1993). Related diseases include Leber's hereditary optic neuropathy (LHON), Parkinson's disease, dystonia, lactic acidosis, various forms of Encephalomyopathies, Huntington's disease and Leigh's disease (Wallace *et al*., 1992). An association with dysfunction of mitochondrial complex I has been reported for most of these diseases.

Mitochondrial complex I, known as NADH-ubiquinone oxdoreductase subunits, is one of five enzyme complexes that functions in oxidative phosphorylation in mammalian mitochondria. Mitochondrial complex I, whose structure is the largest and most complex, is composed of approximately 46 subunits (Hatefi, 1985; Wallance *et al*., 1992; Buchanan and Walker, 1996).

Regarding environmental factors, the results from research have demonstrated that exposure to insecticides including rotenone can induce Parkinson's disease and that activity of the electron transfer system of the mitochondrial complex I of brain, muscle and platelets of Parkinson's patients is reduced. Results were reported from several studies (Mizuno *et al*., 1989; Parker *et al*., 1989; Schapira *et al*., 1989; Cardellach *et al*., 1993; Haas *et al*., 1995; Gorell *et al*., 1998; Menegon *et al*., 1998).

Rotenone is most commonly used as an insecticide and a potent specific complex I inhibitor. Use of rotenone as an insecticide started in 1848 for removal of biting larvae by utilizing a component contained in the legume plant known as Derris and was extracted by Nagai nagayoshi in 1902 as a pure crystalline compound. (Metcalf, 1948). A link between rotenone and NADH oxidase activity of complex I, which can interfere with electron transfer, leading to a gradual decline in use has been reported (Nicklas *et al*., 1985; Ramsay *et al*., 1991).

In recent years, inhibitors specific to mitochondrial complex I including rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydrop-yridine (MPTP) have been reported to induce dopaminergic cell death and inhibit such inhibition. The results clearly demonstrated that the agent can induce Parkinson's syndrome in mammals (Betarbet *et al*. 2000; Manning-Bog *et al*. 2002; Sherer *et al*. 2002).

However, in contrast to mammalian mitochondria where it is presumed that NADH-ubiquinone oxidoreductase can only be detected in mitochondrial respiratory complex I, mitochondrial complex I cannot be detected in Saccharomyces cerevisiae. Involvement of the *NDI1* gene in mitochondrial complex I in a single subunit located in the inner mitochondrial membrane has been reported (Seo *et al*., 1998).

The Yeast NDI1 gene exhibited insensitivity to rotenone, an inhibitor of mitochondorial complex I and the results of the study indicated suppression by Flavone (Yagi, 1991; de Vries and Grivell, 1988; Marres *et al*., 1991). In addition, some studies have reported that expression of NDI1 can occur in transformed cells lacking mitochondrial complex I as a substitute for the function of complex I (Kitajima *et al*., 1998; Seo *et al*., 1998).

Therefore, in this experiment, the recombinant adeno associated virus vector containing the Yeast NDI1 gene was used for expression of DNA and protein in the NDI1 gene in a Mini Pig Neural stem cell line (MPV) having the same heredity as human. Experiments to confirm resistance to rotenone from neural stem cells of minipigs and to confirm its usefulness as a method for gene therapy for treatment of disease induced by defects of mitochondorial complex I including Parkinson's Disease and LHON disease were also performed.

## MATERIALS AND METHODS

### Cell line culture

The cells were isolated from brain tissue of Mini Pig (Micro-pig®, Medi kinetics, South Korea) and a Mini Pig Neural stem cell line (MPV) was used (Choi *et al*., 2016). A large amount of MPV was cultivated using a Hyper Flask (Corning, USA), dispensed into $1\times10^6$ cells / vial, left still in a -20°C freezer for 1 hour and stored carefully in a -80°C deep freezer for future use. The culture medium used for freezing MPV cells is shown in Table 1. DMEM and Ham's F-12 culture solution was diluted 1:1 and 20% FBS, 10% DMSO and 1% P/S were mixed. The frozen cells used in this study were thawed in a 37°C water bath for approximately 3 minutes prior to the experiment, followed by diluting 1:1 with Dulbecco's Phosphate Buffered Saline (DPBS, Hyclone, USA) and centrifuged at 1,500 rpm for 5 minutes. Once the cells had settled, the supernatant was removed and 1 ml of a cell culture medium mixed with 10% FBS and 1% P/S was added to DMEM/Ham's F-12 diluted 1:1 and the cell pellet was released. Cells were counted using an automated cell counter (Logos Biosystems, South Korea) and cells were inoculated with $1\times10^6$ cells/6-3 in 100 mm culture dishes (Corning, Becton Dickinson, USA). After inoculation for 24 hours, it was replaced with a fresh culture solution and subcultured when growth over approximately 80-90% of the area of the culture dish was obtained, using Trypsin 0.25% (Hyclone, USA).

### NDI gene transfection

For transfection of the NDI gene, DMEM/F-12 culture medium and MPV was inoculated in a six-well plate (Corning, USA) at a concentration of $1\times10^5$ cells/3 ml/well, with inoculation of MPV at 37°C for 24 hours, 5% $CO_2$, followed by incubation in an incubator with 95% humidity and attached. After adhesion was confirmed through a microscope, the wells were washed with DPBS for removal of suspended cells and previous culture. After adhesion of MPV cells was confirmed through a microscope, the plate was washed with DPBS for removal of the suspended cells and previous culture. In addition, the plate containing MPV cells was treated with recombinant adeno-associated virus ndi1 (rAAV-ndi1) for transfection, with periodic replacement

**Table 1:** Information on the culture used in the experiment.

| Type | Media | Supplements |
|---|---|---|
| Cell culture | DMEM[1)a] /Ham's F-12[2)] | FBS[3)], P/S[4)] |
| Cell selection | DMEM[1)b] | FBS, P/S, rotenone[5)] |
| Cell-cryopreservation | DMEM[1)a] Ham's F-12 | FBS, P/S, DMSO[6)] |

Information about the media and supplements used in the study.[1)] DMEM: Dulbecco's Modified Eagle's Medium (Welgene, South Korea), [a] contained high glucose (4500 mg/l) in the medium and [b] was without glucose.[2)] Ham's F-12: F-12 Nutrient Mixture, Ham (Welgene, South Korea).[3)] FBS: Fetal Bovine Serum (Hyclone, Laboratories Inc, USA).[4)] P/S: Penicillin/streptomycin (Hyclone, Laboratories Inc, USA).[5)] Rotenone: rotenone (Sigma aldrich, Lours, USA).[6)] DMSO: Dimethyl Sulfoxide (Sigma aldrich, USA).

**Table 2:** Information on the primers used in RT-PCR.

| Primeres | Sequence (5′→3′) | Product length |
|---|---|---|
| GAPDH | Fw: AGGACCAGGTTGTGTCCTGT | 108 |
| | Rv: ACCAGGAAATGAGCTTGACGA | |
| NDI1 | Fw: AGCTCTGCCCATCGTTTTGA | 116 |
| | Rv: CTTCAACTTTGGCGACAGCC | |

Fw: Forward.

Rv: Reverse.

with a cell selection culture solution containing 10% FBS, 1% P/S and 0.2 μM rotenone (Table 1).

### RNA extraction and cDNA synthesis

Following subculture, the experiment was performed for restoration of mitochondrial activity of thawed cells. RNA was extracted from MPV cells and transfection of MPV cells with the *NDI1* gene was performed using Trizol (Invitrogen, USA) when proliferation of approximately 80-90% of the area of a 60mm culture dish was achieved in an incubator at 37°C, 5% $CO_2$ and 95% humidity. RNA extraction was performed according to the protocol for Trizol reagent. The concentration was determined at an absorbance of 260 nm using a microplate reader and the samples were stored in a deep freezer at -80°C. Synthesis of cDNA was performed according to the protocol for the reagent using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA).

### Reverse Transcription PCR (RT-PCR)

Reverse transcription PCR (RT-PCR) was performed to confirm normal expression of the NDI1 gene in MPV cells transfected with the NDI1 gene. 1 μl of the synthesized cDNA and 1 μl of each primer prepared at a concentration of 10 pmol were mixed with RT-PCR buffer and i-pfu polymerase (Intron Biotechnology Inc, South Korea) and the experiment was performed according to the standard protocol for the RT-PCR equipment (Table 2).

### Mitochondria Isolation and Western blot

A commercially available kit (Thermo Scientific® Mitochondria Isolation Kit for Cultured Cells) was used and the isolation protocol was as follows. First, at least $2\times10^7$ cells were prepared, followed by addition of 800 μL of Reagent A and stored on ice for 2 minutes. For the next step, 10 μL of Reagent B was added, followed by vortexing for 5 minutes on ice. As a third step, 800 μl of Reagent C was added, followed by centrifuging at 700 × g for 10 min at 4°C. In the fourth step, the supernatant from step 3 was centrifuged at 3,000×g for 15 minutes at 4°C. As the fifth step, the supernatant from step 4 was removed and the pellet was washed with 500 μL of Reagent C. Finally, centrifuging was performed at 12,000×g for 5 minutes at 4°C. Western blot was performed using extracted Mitochondria. The mitochondrial pellet was washed with PBS (Hyclone Laboratories Inc, South Logan Utah, USA), followed by mixing with 100 μL of RIPA buffer (ThermoFisher, Waltham, USA) containing 1% Protease inhibitor (ThermoFisher, Waltham, USA) and then transferred to a 1.5 mL tube (AXYGEN® Central avenue Union City, USA) and centrifuged at 13,000 rpm for 10 min at 4°C. Measurement of protein concentration was performed using the Bradford and loaded on SDS-PAGE gel. Anti-NDI1 and anti-COX IV were used as primary antibodies and anti-rabbit and anti-mouse HRP (Invitrogen, USA) were used as secondary antibodies. The blocking buffer contained skim milk and BSA. The presence or absence of a protein band was confirmed using a detection regent (ThermoFisher, chemiluminescent substrate, USA).

### Immunofluorescence

Immunofluorescence was performed to determine the presence of the NDI1 protein in the cell. MPV cells and ndi1-infected MPV cells were cultured at approximately $1\times10^5$ cells in confocal dishes, respectively. After 24 hours, attachment of cells was confirmed through an optical microscope and after removal of the culture medium, each cell was fixed for approximately 2 minutes with a cell fixative (acetone: methano, 3: 1 mixture) and stored at -20°C. The cell fixative was removed, followed by washing three times for 7 minutes with DPBS. The primary and secondary antibodies used are shown in Table 3. The primary antibody was stirred at 4°C for 20 hours, followed by washing three times for 7 minutes. The secondary antibody was stirred for 90 minutes in a dark room at room temperature, followed by washing in the same manner. Staining of the cell nucleus was performed using 4'-6-Diamidino-2-phenylindole (DAPI).

### Cell viability measurements

The cell count was used for LUNA and the rate of cell death for rotenone was determined using the MTS [3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay. Using MTS (Promega, USA), cells are treated with a tetrazolium compound on a culture plate containing cells for production of formazan, a purple crystal using a redox reaction and the amount of Formazan was proportional to the amount of cells grown. Proliferation or death of cells can be easily measured. The cells were incubated with rotenone at a concentration of 0.2 mM and the absorbance was compared using the daily MTS assay for three days and the rate of cell death was determined. An optical microscope was used as the imaging equipment. The frozen MTS reagent was thawed in a 37°C water bath for 10 minutes and 20 μl of the reagent was mixed per 100 μl of the culture solution. After reaction for 2 hours in the same environment as the cell culture, it was confirmed by absorbance at 490 nm.

### Statistical analysis

Standard deviation (±SD) and significance test for discrete variables were performed using an SAS program (Statistics Analytical System, version 9.4, USA) and values for the rate of cell death were expressed as percentages. The significance between each treatment group was determined at the 5% level using the Duncan multiple test. A *p* value less than 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

### Expression of the NDI1 gene in MPV cells

RNA from MPV cells cultured at approximately $1\times10^6$ cells and NDI1-infected MPV cells were extracted and gene amplification was performed using cDNA synthesis and RT-PCR to determine whether the NDI1 gene was normally expressed in MPV cells using rAAV-NDI1 vector. The presence of the NDI1 gene was then confirmed by electrophoresis (Fig 1). Formation of a band in the NDI1-infected MPV was confirmed as shown in Fig 1. As shown in Table 2, the NDI1 primer, a reverse transcription reaction using cDNA was performed to confirm normal expression of the NDI1 gene in the NDI1-infected MPV.

RNA was extracted from MPV cells transfected with the NDI1 gene to confirm expression of the Saccharomyces cerevisiae gene (NDI1 gene), which has not been detected in MPV cells, which are mammalian cells. The findings confirmed that expression of the NDI1 gene occurred in MPV cells transfected with the NDI1 gene by synthesis of cDNA and RT-PCR using the NDI1 primer. Park *et al*. (2007) reported that the NDI1 gene was expressed in Leber's hereditary optic neuropathy (LHON) mutant cells and the NDI1 gene was then confirmed by PCR using an NDI1 primer.

### Confirmation of NDI1 protein expression in MPV cells

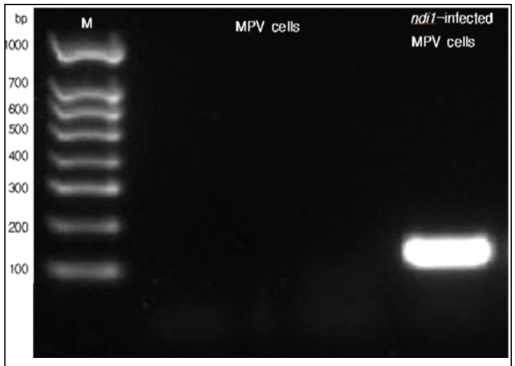
In this experiment, western blot and immunofluorescence staining were performed to confirm normal expression of the NDI1 gene as NDI1 protein and its presence in mitochondria; the results are shown in Fig 2 and 3. As shown in Fig 2, according to the size of the membrane band of anti-cytochrom (COX IV), it was confirmed that mitochondria of MPV cells and MPV cells expressing NDI1 were normally extracted and the experiment was performed using similar amounts. The results also confirmed that the anti-NDI1 membrane band appeared only in MPV cells expressing the NDI1 gene. Immunofluorescence staining was performed to confirm normal expression of the NDI1 gene and its presence in the MPV transfected with the NDI1

**Table 3:** Information on the antibodies used in immunofluorescence.

| Antibodies(Ab) | |
|---|---|
| Primary Ab | Secondary Ab |
| Anti-COX IV[1)], mouse monoclonal | Goat-Anti-Mouse Alexa fluor647 (IgG HandL)[3)] |
| Anti-NDI1[2)], rabbit | Goat-Anti-Rabbit Alexa fluor488 (IgG Fc)[4)] |

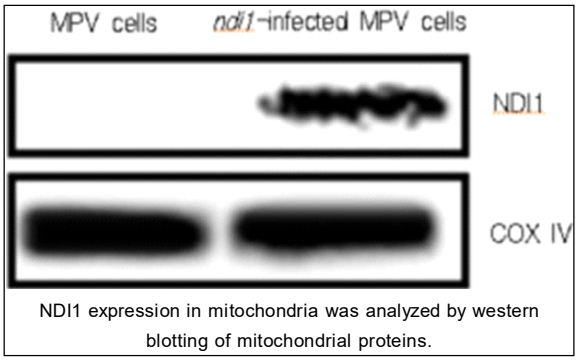
[1). 3), 4)] Abcam, Cambridge, UK.

[2)] The manufactured product was used (Seo *et al*, 2006).




(M: marker, Lane 1: MPV, Lane 2: *ndi1*-infected MPV.

**Fig 1:** Expression of the *ndi1* gene in MPV cells infected with the rAAV-*ndi1*particles by RT-PCR.




NDI1 expression in mitochondria was analyzed by western blotting of mitochondrial proteins.

**Fig 2:** Expression and mitochondrial localization of the NDI1 protein in MPV cells infected with the rAAV-*NDI1* particles by western blot.

gene. Photographs of each MPV and MPV transfected with NDI1 genes, listed in the order of NDI1, mitochondria and cell nucleus are shown in Fig 3, a-c and e-g. Fig 3, d and h show composite pictures. As shown in Fig 3, the results confirmed that fluorescence of NDI1 occurred only in e; these results confirm that normal expression of the NDI1 gene occurs in MPV cells (Seo *et al*., 2000; Jason *et al*., 2007; Santidrian *et al*., 2013).

Western blot and immunofluorescence staining were performed to confirm expression of the NDI1 gene in MPV cells and in mitochondria. The results confirmed that expression of the NDI1 gene occurred in MPV cells and was detected in mitochondria. These results were the same as those reported by Park *et al*. (2007), and expression of the NDI1 gene as a protein and its detection in mitochondria was reported (Seo, 1998; Nguyen *et al*., 2017; Jin *et al*., 2017). In addition, it was also confirmed that the band of anti-NDI1 appeared only in MPV cells expressing NDI1, confirming the same results as those reported by Park *et al*. (2007).

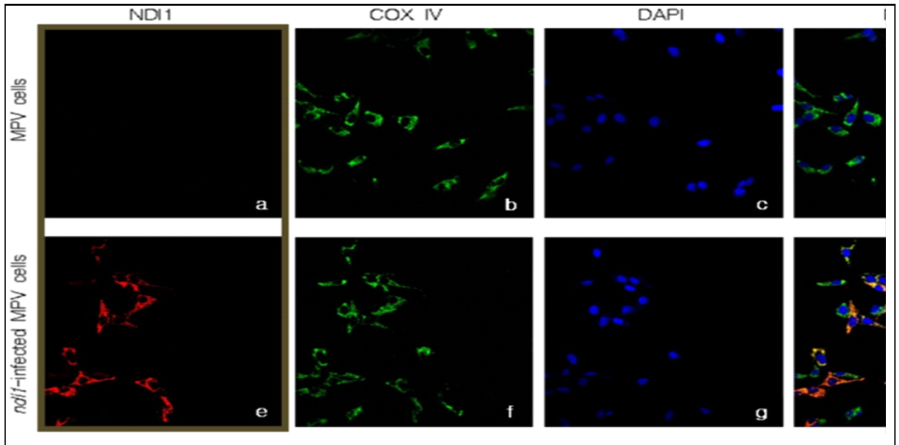
### Confirmation of apoptosis caused by Rotenone

In this experiment, apoptosis caused by rotenone was confirmed using an MTS assay. MTS, a color developing reagent that utilizes the redox reaction of cells, can reduce formazan with purple crystals after treatment of cells with tetrazolium, in proportion to the proliferation of cells; thus, proliferation and death of cells can be easily studied. The MTS assay was performed daily for three days after attachment of cells to the culture plate and the results are shown in Table 4. The absorbance determined using the MTS assay was expressed as a percentage of the value for the group not treated with rotenone and the value for the treated group. Following treatment with rotenone, on day 1, MPV cells died at 54.81% and NDI1-infected MPV cells at 34.14%, respectively, on day 2, at 58.77% and 33.21%, respectively. On the third day, all MPV cells died and only 28.36% of MPV cells transfected with the NDI1 gene died. The absorbance was significantly higher for MPV cells not treated with rotenone compared with treated and the same results were observed in MPV cells transfected with the NDI1-gene. The color of the reagent changed to purple as formazan was reduced in each MPV cell, with high absorbance, so that cell death increased after treatment with rotenone. Comparison of MPV cells showed significantly more NDI1-infected MPV cells compared with MPV cells in both experimental groups treated with or without rotenone. MPV cells transfected with the NDI1 gene showed greater viability than MPV cells that were not transfected (Table 4). The mortality rate for Rotenone-treated MPV cells and NDI1-infected MPV cells for three days was confirmed, as shown in Fig 4. After culturing the same amount of MPV cells on the first day, on the first day of treatment with rotenone, a tendency to decrease was observed for both cells, although the graph showed a gentler slope for NDI1-infected MPV. From the second day, the number of NDI1-infected MPV cells was maintained until the third day. However, for MPV not treated with the NDI1 gene, the number of cells showed a rapid decrease and died until the third day. These results could be clearly confirmed by observation using an optical microscope as shown in Fig 5.

**Table 4:** Cell death rate during rotenone treatment for three days.

| Culture time (days) | Groups | |
|---|---|---|
| | MPV cells (%) | *ndi1*-infected MPV cells (%) |
| 1 | 54.81$^{a}$±(2.26) | 34.14$^{b}$±(3.95) |
| 2 | 58.77$^{a}$±(2.48) | 33.21$^{b}$±(2.81) |
| 3 | 100.00$^{a}$±(0.96) | 28.36$^{b}$±(1.84) |

Each value was mean ± SDMeans with different superscript within the rows were significantly different ($p$< .05).




NDI1, Mitochondria and nuclear staining of MPV cells (a - c) and *ndi1*-infected MPV (e - g). (d, h) are merged images. NDI1 was observed into NDI1-infected cells, not in the un-infected cells (a, e).

**Fig 3:** Expression and mitochondrial localization of the NDI1 protein in MPV cells infected with the rAAV-*NDI1* particles by immunofluorescence.

According to a study reported by Marella *et al*. (2007), approximately 70% of dopamine cells (PC12) had died four days after treatment with 1 μM rotenone, although approximately 20% of the NDI1 transfected dopamine cells were dead. However, the results were similar to those of this study. However, after treatment with 0.2 μM rotenone for four days , the rate of apoptosis of dopamine cells and PC12 cells, was approximately 10% and the rate of death for dopamine cells transfected with the NDI1 gene (PC12-NDI1) was estimated as approximately 1%. In addition, according to a study reported by Seo *et al*. (1999), who reported on the death rate for 0.1 μM rotenone in HEK 293

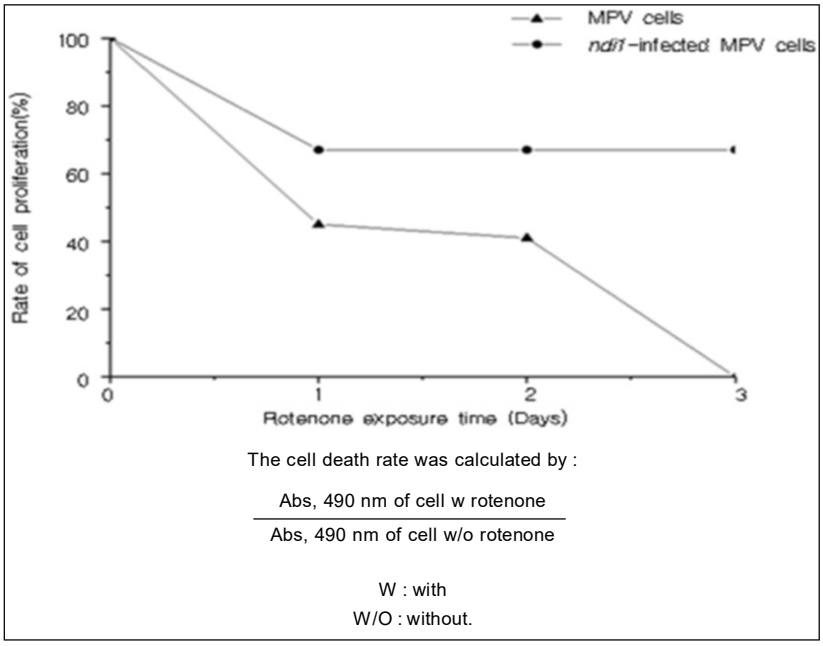
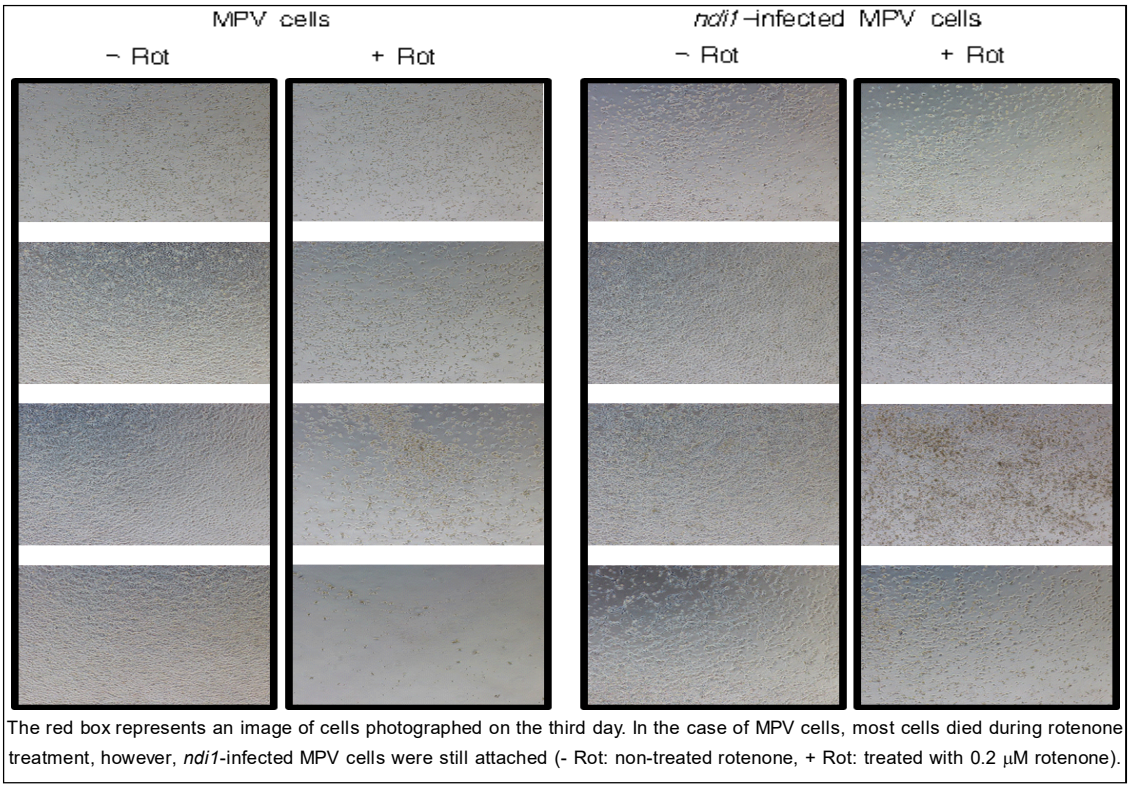



**Fig 4:** Death rate quantification of un-infected and *ndi1*-infected MPV cells after rotenone treatment.




The red box represents an image of cells photographed on the third day. In the case of MPV cells, most cells died during rotenone treatment, however, *ndi1*-infected MPV cells were still attached (- Rot: non-treated rotenone, + Rot: treated with 0.2 μM rotenone).

**Fig 5:** Observation of apotosis by rotenone treatment for three days using an optical microscope.

cells that almost died on the fourth day, but increased by approximately three times more than the first in HEK 293 cells transfected with the NDI1 gene. Almost all apoptosis occurred on days 3-4 in cells that were not transfected with the NDI1 gene, similar to previous studies. However, proliferation in HEK 293 cells was observed for cells transfected with the NDI1 gene (Seo *et al*., 1999). The findings suggest that resistance to rotenone exposure can show minor variation depending on the cell type.

In this experiment, transfection of rAAV-NDI1 into the Mini Pig Neural stem cell line (MPV), known to have a genotype similar to humans, with the NDI1 gene, which is insensitive to rotenone, known to cause Parkinson's disease, was attempted. Normal expression of the NDI1 gene in MPV cells was confirmed by Western blot and immunofluo rescence staining.

To determine whether the NDI1 gene can function normally after treatment with rotenone, 0.2 μM of rotenone was applied with incubation for three days and the rates of cell death were assessed every 24 hours using an optical microscope and an MTS assay. According to the results, NDI1-infected MPV cells showed resistance in the rotenone environment and survived more than 70% on the third day. These results were similar to those of complex I defects in human cells reported by Seo *et al*. (2000). Seo *et al*. (2000) and Marella *et al*. (2007) reported that mitochondrial defects due to genetic and environmental factors as the cause of cell death and oxidative stress can damage DNA and induce cell death. According to Marelle *et al*. (2007), as a mechanism of apoptosis and DNA damage, blockage of the electron transport pathway of complex I by rotenone can cause a rapid increase in the levels of ROS within a few hours and ROS can directly induce apoptosis and DNA damage. However, phosphorylation of the kinase can be induced by p38 kinase and JNK.

Activation of the kinase pathway has been linked to the mechanism of mitochondrial apoptosis through phosphorylation of BAX, resulting in release of apoptosis progenitors including AIF and Endo G. Another study reported that in mitochondria, release of Cyt C occurred earlier than the release of AIF/Endo G and was involved in activation of Caspase 9. Therefore, it is thought that the cause of MPV cell death was defects of complex I caused by rotenone and generation of ROS caused by blockage of the electron transport system.

## CONCLUSION

Based on these experimental findings, the NDI1 gene can be used in stem cells as a method of gene therapy for treating disease caused by mitochondrial deficiency, particularly Parkinson′s disease LHON. In addition, expressing the NDI1 gene in sperm of rodents can confirm the activity of ROS of gene-expressed sperm and whether NDI1 gene-expressed sperm are inherited in rat offspring.

## ACKNOWLEDGEMENT

We thank Dr. Takao Yagi at TSRI for kindly providing the *NDI1* antibody. This research was supported by the Daegu University Research Grant 2020, Republic of Korea.

### Conflict of interest

The authors have declared that no competing interests exist.

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