Nicotinamide Riboside Attenuated Cell Damage Induced by the MT-ND4 (G11778A) Mutation

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Research Article

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Abstract

The mechanisms underlying cellular damage induced by the m.11778G > A variant in complex I of Leber Hereditary Optic Neuropathy (LHON) remain critically important. Currently, though no curative drug therapy is available for LHON, mitochondria-targeted therapies represent promising therapeutic avenues. Nicotinamide riboside (NR), a precursor to NAD+, enhances mitochondrial respiration and reduces oxidative stress, positioning it as a candidate for LHON treatment. To elucidate the specific effects of NR on cell death triggered by the R340H mutation, mitochondrial dysfunction was investigated in 661W photoreceptor cells expressing exogenous MT-ND4 (m.11778G > A). The study revealed that NR mitigated cell death and apoptosis in these cells under galactose conditions and ameliorated pattern ERG impairments in MT-ND4 mice. Mechanistically, NR treatment attenuated the reduction in oxygen consumption rate (OCR) and the increase in reactive oxygen species (ROS) levels in 661W cells carrying the R340H mutation. Furthermore, cells expressing MT-ND4 (m.11778G > A) exhibited decreased catalase (CAT) activity, superoxide dismutase (SOD) levels, and glutathione disulfide (GSSG), all of which were restored by NR administration. Collectively, these findings indicate that the MT-ND4 (m.11778G > A) mutation promotes cellular death through disrupting oxidative balance, as evidenced by impaired mitochondrial oxygen consumption, elevated oxidative products, and weakened antioxidant capacity. NR appears to confer rescue effects by restoring oxidative equilibrium, underscoring it as a potential therapeutic approach for LHON.

Highlights

- Nicotinamide riboside (NR) has first been demonstrated to provide significant protection against the toxic effects associated with the ND4 (m.11778G>A) mutation.
- This ND4 mutation primarily disrupts redox balance, thereby impairing mitochondrial respiration, increasing oxidative damage, and reducing antioxidant defenses.
- NR appears to rescue function by restoring oxidative equilibrium, highlighting its promise as a therapeutic strategy for LHON.

1. Introduction

Leber Hereditary Optic Neuropathy (LHON) is an inherited mitochondrial disorder characterized by degeneration of the optic nerve, leading to severe visual impairment, irreversible blindness, most frequently in young adult males [1]. The disease is predominantly associated with pathogenic mutations in mitochondrial DNA (mtDNA) with the G-to-A transition at mitochondrial nucleotide 11778 (m.11778G > A) in the NADH dehydrogenase subunit 4 (ND4) gene of complex I accounting for more than half of all LHON cases [2]. This mutation results in an arginine to histidine substitution at position 340 (p.R340H) and is particularly associated with severe visual loss and poor recovery, making it a major focus of numerous studies of pathophysiological and therapeutic research [3–6].

The R340H mutation is a key pathological factor in LHON with mitochondrial dysfunction due to this mutation playing a central role in disease progression. Although gene therapy approaches are currently under clinical investigation, their long-term efficacy and safety profiles remain uncertain [7]. Although there are no curative drug therapies for LHON, alternative strategies targeting mitochondrial function continue to be of a promising therapeutic strategy for LHON.

Emerging evidence indicates that nicotinamide riboside (NR), a precursor of nicotinamide adenine dinucleotide (NAD^+), can effectively elevate NAD^+ levels in highly metabolic tissues. NAD^+ serves as an essential coenzyme for several NAD^+-dependent enzymes involved in mitochondrial oxidative phosphorylation and energy metabolism [8]. Supplementation with NR has demonstrated potential in attenuating oxidative stress and mitigating bioenergetic deficits, positioning it as a candidate for rescuing cellular damage in mitochondrial disorders. Given the central roles of oxidative stress and mitochondrial dysfunction in LHON pathogenesis, NR may alleviate, halt, or even reverse the pathological processes triggered by the m.11778G > A (p.R340H) mutation. Since early disease mechanisms—including electron transport chain deficiency, redox imbalance, and compromised antioxidant capacity—are sensitive to intervention and provide critical insight into mitochondrial failure, this study aims to investigate the effects of NR on oxidative stress and cellular damage induced by the R340H mutation in LHON.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM, high glucose; BIOEXPLORER, Cat#B1101-001); DMEM glucose-free medium (Basalmedia, Cat#L160KJ); Fetal Bovine Serum (FBS; Capricorn, Cat#F12A); Penicillin and Streptomycin (Gibco, Cat#15140122); Galactose (Sigma-Aldrich, Cat#G5388); Puromycin (Basalmedia, Cat#S250J0); Nicotinamide Riboside (NR; Yuanye, Shanghai, China, Cat#S31692); Reactive Oxygen Species (ROS) Assay Kit (Solarbio, Beijing, China, Cat#CA1410); Superoxide Dismutase (SOD) Assay Kit with WST-8 (Beyotime, Cat#S0101S); Catalase (CAT) Assay Kit (Solarbio, Cat#BC0205); GSH and GSSG Assay Kit (Beyotime, Cat#S0053); DAPI (Beyotime, Cat#C1005); Hoechst 33342 (Solarbio, Cat#C0030); Seahorse XFe24 Microplates (Agilent, Cat#02340); Agilent Seahorse XF DMEM Medium (Agilent, Cat#103015); Sodium Pyruvate (Agilent, Cat#103578); Glutamine (Agilent, Cat#103579); D-Glucose (Agilent, Cat#103577); BCA Protein Assay Kit (Beyotime, Cat#P0010).

2.2. Adeno-Associated Viral Vectors

The AAV2/2 vectors used in these studies were generated with all constructs driven by the cytomegalovirus immediate early gene enhancer and the chicken beta-actin promoter. The mutant human R340H ND4 (MT-ND4) construct, which was described previously, was designated AAV2.MT-ND4.HA. This construct incorporates the ATPc mitochondrial targeting sequence to facilitate the shuttle

of the allotopically expressed protein into mitochondria. Additionally, a C-terminal HA tag was included for immunodetection purposes.

2.3. Culture of 661W Cells and stable expression of exogenous ND4 gene

661W photoreceptor cells were cultured according to the established protocol. To study the cytotoxic effects of the MT-ND4, we utilized allotopic expression of both the MT-ND4 subunit of complex I and the wild-type ND4 (WT-ND4). Allotopic expression refers to the strategy of expressing a nuclear-encoded version of a typically mitochondrial DNA-encoded gene in the cytoplasm, followed by its import into the mitochondria. The ATP synthase subunit c (ATPc) mitochondrial targeting sequence (MTS) was employed to facilitate the mitochondrial import of the allotopically expressed R340H and HA-tagged polypeptides. As a result, the recoded R340H mutant or wild-type ND4 gene product had been successfully imported into the mitochondria [9–13]. For the experiments, 661W cells at approximately 50%-70% confluence were infected with 5.0×10^8 lentiviral particles carrying the ND4 gene with the MT-ND4 gene or the WT-ND4 gene in high-glucose medium. After 72 hours, the cells were selected by culturing in medium containing 0.3 μg/mL puromycin for two weeks to enrich for populations stably expressing the exogenous ND4. Cell viability was subsequently assessed using a Leica DM500 biological microscope.

2.4. Analysis of apoptosis via DAPI Staining

To evaluate the impact of exogenous ND4 expression and NR treatment on apoptosis in 661W cells, the cells were cultured under various conditions for three days and subsequently subjected to a DAPI staining-based apoptosis assay. Briefly, after the treatment period, cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 for 15 minutes. The cells were then stained with 300 nM DAPI solution at 37°C for 5 minutes in the dark. Following staining, the samples were washed three times with phosphate-buffered saline (PBS). Excess liquid was carefully removed, and the coverslips were mounted using an antifade mounting medium. Imaging was performed using a Leica DMi8 fluorescence microscope. The number of apoptotic cells was quantified based on characteristic nuclear morphology (condensation and fragmentation) using Image J software, and the apoptotic rate was expressed as the percentage of apoptotic cells relative to the total number of cells counted.

2.5. Assessment of Mitochondrial Respiration in 661W Cells [14]

Mitochondrial function was assessed by measuring the oxygen consumption rate (OCR)—a key parameter of mitochondrial respiration—using a Seahorse XFe24 Analyzer (Agilent). 661W cells expressing different exogenous ND4 genes were plated at a density of 8×10^3 cells per well in Seahorse XFe24 microplates andcultured for 24 hours in either high-glucose DMEM or glucose-free DMEM supplemented with galactose. Sensor cartridges were hydrated overnight at 37°C in a non-CO_2 incubator with Seahorse XF calibrant solution. Prior to the assay, the culture medium was replaced with Agilent

Seahorse XF DMEM medium supplemented 2 mM glutamine, 1 mM D-glucose, and 1 mM sodium pyruvate. Following the medium change, the cells were incubated at 37°C for 1 hour to allow temperature and PH equilibration before initiating OCR measurements. OCR measurements were taken after the sequential injection of FCCP (1.5 μM), Rot/AA (0.5 μM), and oligomycin (1.5 μM). Protein concentrations were determined using a BCA protein assay kit, and OCR values were normalized to the total protein content. The entire procedure was conducted at 37°C.

2.6. Measurement of Reactive ROS in 661W cells [15]

661W cells expressing different exogenous ND4 variants were seeded into 12-well plates. After specific treatments with or without NR, the cells were stained with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and incubated at 37°C for 30 minutes. Subsequently, the nuclei were stained with Hoechst 33342 dye for 15 minutes. Following two washes with PBS, ROS levels were assessed using fluorescence microscopy.

2.7. Detection of Oxidative Markers (SOD, CAT, and GSSG) [15]

Cells expressing either WT-ND4 or MT-ND4 were seeded into a 96-well plate and incubated in a 5% CO_2 incubator, with or without NR treatment. After 72 hours of incubation, the culture medium was removed, and cells were washed twice with PBS. The superoxide dismutase (SOD) activity was determined using a Total Superoxide Dismutase Assay Kit. Catalase (CAT) activity was measured with a CAT Assay Kit, and the level of oxidized glutathione disulfide (GSSG) was quntified using a GSH/GSSG Assay Kit, in accordance with the manufacturer's instructions.

2.8 Animals and Injection Procedures

Adult male DBA/1J mice were purchased from GemPharmatech Co. Ltd and housed under standard laboratory conditions with a 12-hour light/12-hour dark cycle. All experimental procedures were approved by the Animal Care and Veterinary Service of Fujian Medical University and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male mice aged 10 to 12 weeks (N = 20) were used in this study. Intravitreal injections were performed in the eye using 1 μL of AAV2/2.MT-ND4.HA (1×10^9 total viral particles at a concentration of 10^{12} VG/mL) and an equal dose of AAV2/2.WT-ND4. The NR treatment was performed as described in our previous report [16]. Mice were given 12 mM NR in their drinking water ad libitum, while the control groups received drinking water without NR. Water bottles were changed twice a week. Ocular tissues and optic nerves were collected 4.5 months after ND4 injection.

2.9. Pattern ERG(PERG)

Pattern electroretinograms (PERGs) were recorded using an Espion E^2 system (Diagnosys, Inc., Lowell, MA). Mice were anesthetized with a combination of ketamine (50 mg/kg) and medetomidine (1 mg/kg) and positioned on an elevated platform maintained at 37°C, placed 20 cm from the stimulus monitor. The monitor displayed 200 mm interspaced black and white bars at 50 cd/m2, 0.05 cycles/deg, 100%

contrast, and 2 Hz temporal frequency. A 2-mm diameter gold loop electrode was placed on the undilated pupil facing the screen. A total of 1800 traces were recorded (two consecutive sequences of 900 traces, sample frequency 1000 Hz, sweep duration of 10 ms before stimulus to 400 ms after stimulus). Retinal signals were amplified (10,000 fold) and a band-pass filter of 0.3 to 300.0 Hz was used. The reference stainless steel needle electrode was placed in the scalp and the ground electrode was placed in the tail. Final waveforms were scored by removing all outlier traces above + 30 μV and below − 30 μV.

2.10. Statistical Analysis

All experiments were repeated at least three times independently. Data are expressed as mean ± standard deviation. Statistical analyses were carried out using GraphPad Prism software (version 10.0; San Diego, CA, USA). Comparisons between two groups were performed using Student's t-test. For comparisons among three or more groups, one-way analysis of variance (ANOVA) was applied, followed by the least significant difference (LSD) post hoc test. A p-value of less than 0.05 was considered statistically significant.

3. Result

3.1 Impact of NR on cellular morphology in the context of MT-ND4 expression

To assess the potential protective effect of NR against MT-ND4-induced cellular damage, we examined morphological changes in 661W cells expressing either WT-ND4 or MT-ND4 using light microscopy. Cells were cultured in DMEM containing-galactose (without glucose) for 72 hours to enforce dependence on mitochondrial metabolism. As shown in Figs. 1A and 1B, cells expressing MT-ND4 exhibited sparse growth and reduced confluency compared to those expressing WT-ND4, indicating impaired survival under metabolic stress. However, supplementation with NR at concentrations of 1 mM, 3.5 mM, 7 mM, or 14 mM significantly improved cell density in cultures expressing the m.11778G > A mutant. Notably, the group treated with 7 mM NR demonstrated the most pronounced rescue effect, with a substantial increase in cell density compared to the MT-ND4 group. These observations suggest that NR attenuates the cytotoxic effects associated with the MT-ND4, thereby supporting its role in promoting cell survival under conditions of mitochondrial dysfunction.

$^{**}P < 0.01$; $^{***}P < 0.001$.

3.2 Effect of NR on MT-ND4-Induced cellular apoptosis

NR supplementation improved cellular density in 661W cells expressing the m.11778G > A ND4 mutation cultured in galactose medium. To further evaluate whether NR confers protection against MT-ND4-induced cellular apoptosis, we performed an apoptosis assay using DAPI staining to assess nuclear condensation. As shown in Figs. 2A and 2B, 661W cells expressing exogenous ND4, whether wild-type or

mutant (m.11778G > A), were cultured in galactose-containing and glucose-free DMEM for 72 hours and subsequently stained with DAPI. Cells expressing MT-ND4 displayed pronounced nuclear condensation compared to WT-ND4-expressing cells, indicative of elevated apoptosis. NR treatment at 1 mM, 3.5 mM, 7 mM, or 14 mM reduced apoptosis in MT-ND4-expressing cells, with 7 mM NR producing the most robust effect, significantly lowering nuclear condensation relative to untreated and other NR-treated groups.

3.3 Influence of NR on mutant ND4 under oxidative stress

ROS, generated as byproducts of mitochondrial electron transport chain reactions, are central mediators of oxidative stress. To investigate the influence of NR on ROS induced by MT-ND4, intracellular ROS levels were evaluated in 661W cells expressing WT-ND4 or MT-ND4. As demonstrated in Figs. 3A and 3B, 661W cells expressing MT-ND4 cultured in galactose medium exhibited higher ROS levels compared to WT-ND4-expressing cells under the same conditions. Treatment with 7 mM NR markedly reduced ROS levels in MT-ND4-expressing cells. These findings suggest that the m.11778G > A mutation aggravates oxidative damage in galactose-induced stress, which can be effectively mitigated by NR intervention.

3.4 Influence of NR on MT-ND4 under anti-oxidative stress conditions

To investigate the protective role of NR against oxidative stress in cells expressing MT-ND4, we measure the levels and activities of key antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), and glutathione (GSSG). As illustrated in Figs. 4A–C, MT-ND4-expressing cells cultured in galactose-containing and glucose-free DMEM medium exhibited significantly lower CAT (A), SOD (B), and GSSG (C) compared to WT-ND4-expressing cells. Notably, treatment with 7 mM NR markedly restored the activity and expression of these antioxidant enzymes. These results indicate that the ND4 m.11778G > A mutation exacerbates oxidative stress, particularly under energy-deficient conditions such as galactose culture, whereas NR supplementation effectively restores redox homeostasis by boosting antioxidant defense mechanisms.

3.5 NR enhanced mitochondrial respiration in cells harboring m.11778G > A mutation

ND4, a mitochondrially encoded subunit of respiratory Complex I, is essential for cellular energy metabolism and ATP generation through oxidative phosphorylation. To evaluate mitochondrial respiratory function, we measured oxygen consumption rate (OCR) using a Seahorse XFe96 extracellular flux analyzer. The oxidative phosphorylation (OXPHOS) capacity was determined through real-time OCR assessments following the sequential injection of oligomycin (an ATP synthase inhibitor), FCCP (a mitochondrial uncoupler), and a combination of rotenone and antimycin A (inhibitors of the electron transport chain). As depicted in Figs. 5A-F, cells harboring m.11778G > A mutation in the ND4 gene and cultured in galactose-containing and glucose-free DMEM medium exhibited the lowest basal and

maximal OCR, as well as reduced mitochondrial ATP production. Treatment with 7 mM NR markedly enhanced both basal and maximal OCR in these cells harboring m.11778G > A mutation compared to untreated controls. These findings indicate that the m.11778G > A mutation impairs mitochondrial respiration, whereas NR supplementation restores respiratory capacity, highlighting its protective role in preserving mitochondrial function.

(A and B) Representative OCR profiles: (A) schematic, (B) 661W cells; (C) basal OCR; (D) mitochondrial ATP production in different groups; (E) maximal respiration; (F) spare respiratory capacity. n = 4. Statistical significance was determined by one-way ANOVA. *P< 0.05; **P< 0.01; ***P< 0.001.

3.6 NR improved the optic nerve and RGC function

To evaluate whether nicotinamide riboside (NR) preserves retinal ganglion cell (RGC) function in vivo, we performed pattern electroretinogram (PERG) recordings, a sensitive and non-invasive method for assessing RGC-driven electrical responses. PERG is particularly valuable in models of LHON, as functional decline in RGCs precedes overt structural degeneration of the optic nerve. As shown in Fig. 6, at 4.5 months post-injection, MT-ND4 animals displayed markedly impaired PERG waveforms, with reduced N2 amplitudes compared with wild-type controls, consistent with progressive RGC dysfunction caused by the m.11778G > A mutation. Importantly, NR supplementation substantially ameliorated these deficits. MT-ND4 animals treated with NR exhibited PERG waveforms that more closely resembled those of wild-type animals, along with significantly larger N2 amplitudes relative to untreated MT-ND4 mice.

These findings indicate that NR treatment preserves functional integrity of the optic nerve and protects RGCs from mitochondrial dysfunction–induced damage. Given that PERG decline is considered a translationally relevant marker of RGC impairment in both experimental models and patients, the observed improvement suggests that NR confers clinically meaningful neuroprotection. Together, these results highlight NR as a promising therapeutic approach for preventing RGC dysfunction and vision loss in LHON.

4. Discussion

Mitochondrial dysfunction induced by the mG11778A (R340H) mutation in the ND4 subunit of complex I is a well-established pathological feature of Leber's Hereditary Optic Neuropathy (LHON). In this study, allotopic expression of the R340H mutant protein—decoded by the m.11778G > A mitochondrial DNA variant—exerted pro-death effects, leading to cellular damage characterized by morphological alterations and apoptosis. Notably, treatment with NR counteracted these deleterious effects and promoted cellular rescue. Among the tested concentrations, NR at 7 mM demonstrated the most robust protective activity.

Given the central role of oxidative stress in LHON pathogenesis, we further investigated the influence of NR on key mitochondrial functional parameters, including mitochondrial oxygen consumption rate. Remarkably, NR enhanced ATP production by preserving ETC integrity and supporting mitochondrial bioenergetics. Additionally, under galactose and free-glucose culture conditions—which impose a

metabolic dependency on mitochondrial respiration—cells expressing the ND4 R340H mutation exhibited a significant reduction in antioxidant defense markers, including catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH), concomitant with elevated reactive oxygen species (ROS) levels. NR treatment effectively attenuated the decline in antioxidant capacity and suppressed ROS accumulation under these conditions. These findings suggest that NR mitigates the pathogenic effects of the R340H mutation by improving mitochondrial function and restoring redox homeostasis. Finally, the protective effect of NR on optic nerve function was corroborated using ERG, which revealed enhanced retinal ganglion cell signaling and supported its potential therapeutic utility in LHON.

Mitochondrial dysfunction represents a core pathological feature of LHON, and although no definitive cure currently exists, targeting mitochondrial pathways has emerged as a promising therapeutic strategy [17]. NR, a precursor of nicotinamide adenine dinucleotide (NAD^+), is of particular interest due to its role in sustaining NAD^+ levels, a crucial cofactor in all cell types. NAD^+ plays a fundamental role in mitochondrial energy metabolism by accepting electrons from NADH to supply the electron transport chain (ETC), thereby driving oxidative phosphorylation and facilitating the conversion of ADP to ATP. As a naturally occurring NAD^+ booster, NR has shown potential as a nutritional supplement in mitigating various metabolic and age-related disorders linked to mitochondrial dysfunction, such as Alzheimer's disease [17, 18].

In this study, the R340H mutation in the ND4 subunit (decoded by the m.11778G > A variant) induced severe mitochondrial impairment, manifested as ETC dysfunction, reduced NAD^+ availability, and disrupted redox homeostasis. Notably, NR administration effectively rescued these deficits, restoring mitochondrial function and rebalancing oxidative stress. These findings highlight the compelling neuroprotective potential of NR in the context of LHON associated with the m.11778G > A variant.

Beyond its canonical role in energy metabolism, NAD^+ also serves as an essential cosubstrate for several enzymatic pathways [19]. Notably, it modulates the activity of sirtuins (e.g., SIRT1 and SIRT3) and ADP-ribosyltransferases [20], which are involved in critical processes such as gene expression, DNA repair, and metabolic regulation. Previous studies have demonstrated that NR supplementation can activate SIRT1 and SIRT3, leading to enhanced oxidative metabolism and reduced oxidative stress [21, 22] However, whether NR exerts its protective effects in LHON specifically through sirtuin-mediated or ADP-ribosylation-dependent pathways remains unclear and warrants further investigation.

In conclusion, the R340H mutation in ND4 is a key driver of LHON pathology, promoting cellular damage and apoptosis via ETC disruption and oxidative stress. NR demonstrates significant protective effects by counteracting these imbalances, suggesting its potential as a therapeutic candidate for LHON. Future studies should focus on elucidating the precise molecular mechanisms, including involvement of sirtuin and ADP-ribosyltransferase pathways, to facilitate the development of effective NAD^+-based therapies for this disorder.

Abbreviations

CAT catalase

DCFH-DA 2',7'-dichlorodihydrofluorescein diacetate

ETC Electron transport chain

GSSG glutathione disulfide

LHON Leber Hereditary Optic Neuropathy

MTS Mitochondrial targeting sequence

MT-ND4 Mutant ND4

NAD^+ Nicotinamide adenine dinucleotide

NR Nicotinamide riboside

OCR Oxygen consumption rate

PERG Pattern electroretinograms

RGCs Retinal Ganglion Cells

ROS Reactive oxygen species

SOD Superoxide dismutase

WT-ND4 Wild-type ND4

Declarations

Author Contributions:

Conceptualization, J.H. and T.H.; writing—original draft preparation, L.F. and M.Y.; writing—review and editing, K.F. and D.Q.; supervision, J.H. and T.H.; funding acquisition, L.F. and T.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement:

All animal experiments were performed in strict accordance with the Institutional Guidelines of Fujian Medical University and were reviewed and approved by the Institutional Animal Care and Use Committees of Fujian Medical University. (approval number: IACUC FJMU 2022-0477)

Informed consent Statement:

Not applicable.

Data Availability Statement:

The datasets used and/or analyzed the current study are available from the corresponding author on reasonable request.

Conflicts of Interest:

There are no conflicts of interest.

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Figures

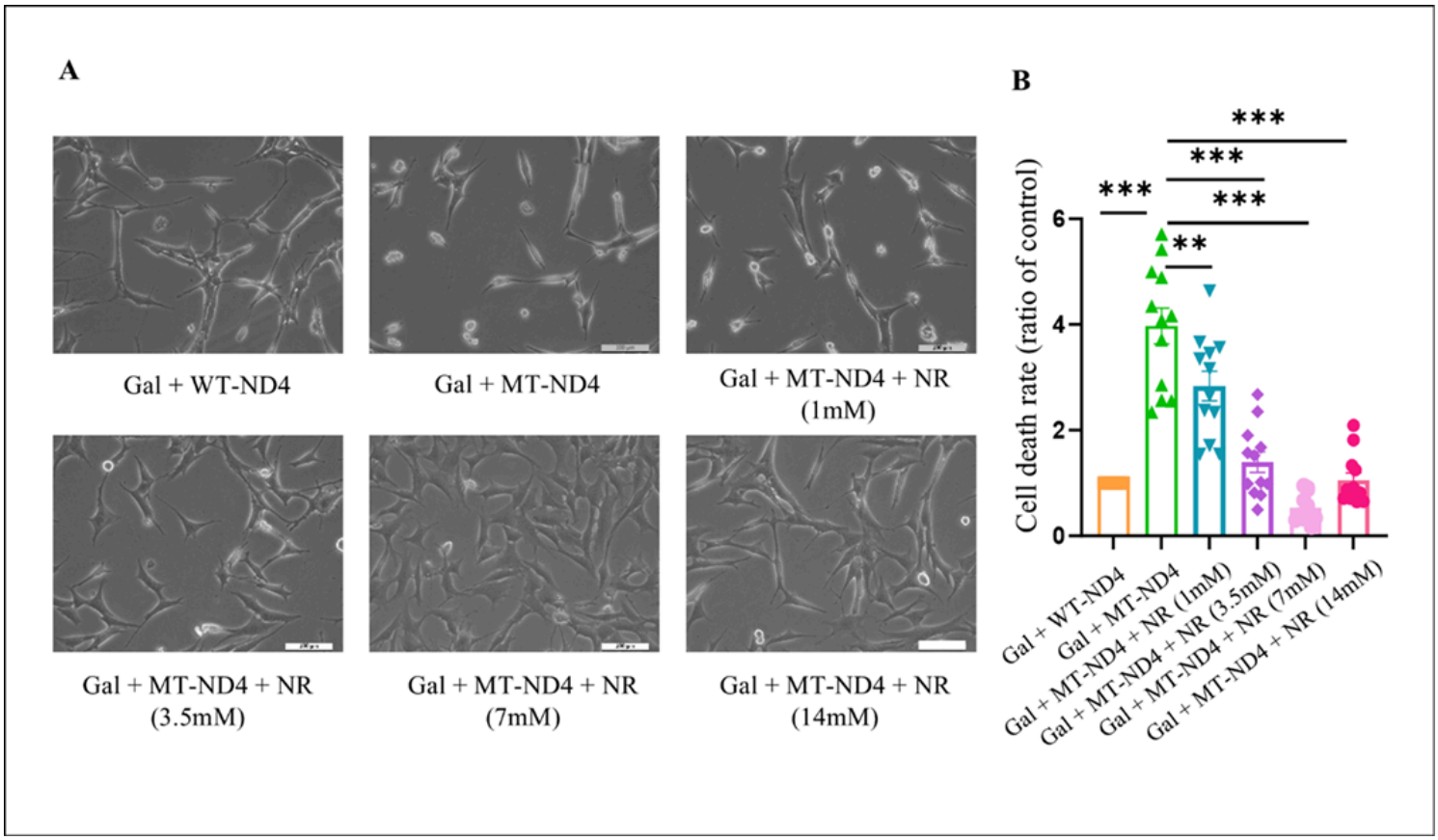

Figure 1

NR treatment ameliorated the impaired cellular morphology in 661W cells expressing the MT-ND4 (m.11778G>A). 661W cells expressing exogenous ND4 (wild-type or m.11778G>A mutant) were cultured in galactose-containing medium. Cells were subjected to the indicated treatments. (A) Representative light microscopy images of cellular morphology. (B) Quantification of cell morphology presented as statistical analysis. Scale bar = 100 μm. Data are presented as means ± SEM, n ≥ 4. Statistical significance was determined by one-way ANOVA:

**P< 0.01; ***P < 0.001.

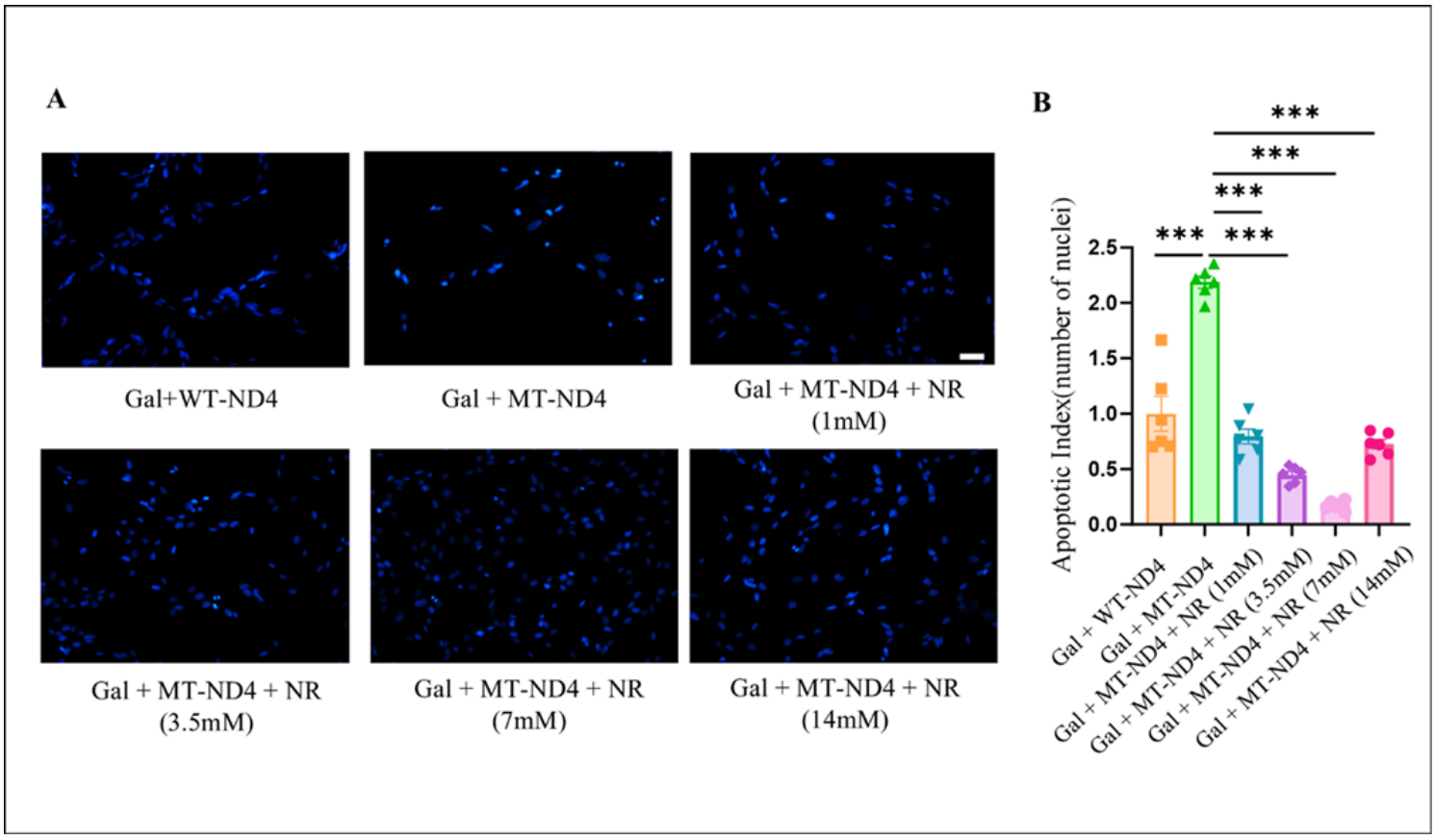

Figure 2

Effect of NR on MT-ND4-Induced cellular apoptosis. 661W cells expressing exogenous wild-type or mutant (m.11778G>A) ND4 were cultured in galactose-containing and glucose-free DMEM medium. Following respective treatments, cells were stained with DAPI and visualized using fluorescence microscopy (A). Apoptotic cells were quantified based on nuclear condensation and fragmentation observed in DAPI-stained samples, with statistical results shown in (B). Scale bar = 100 μm. Data represent means ± SEM; n ≥ 4. Statistical significance was determined by one-way ANOVA. ***P < 0.001.

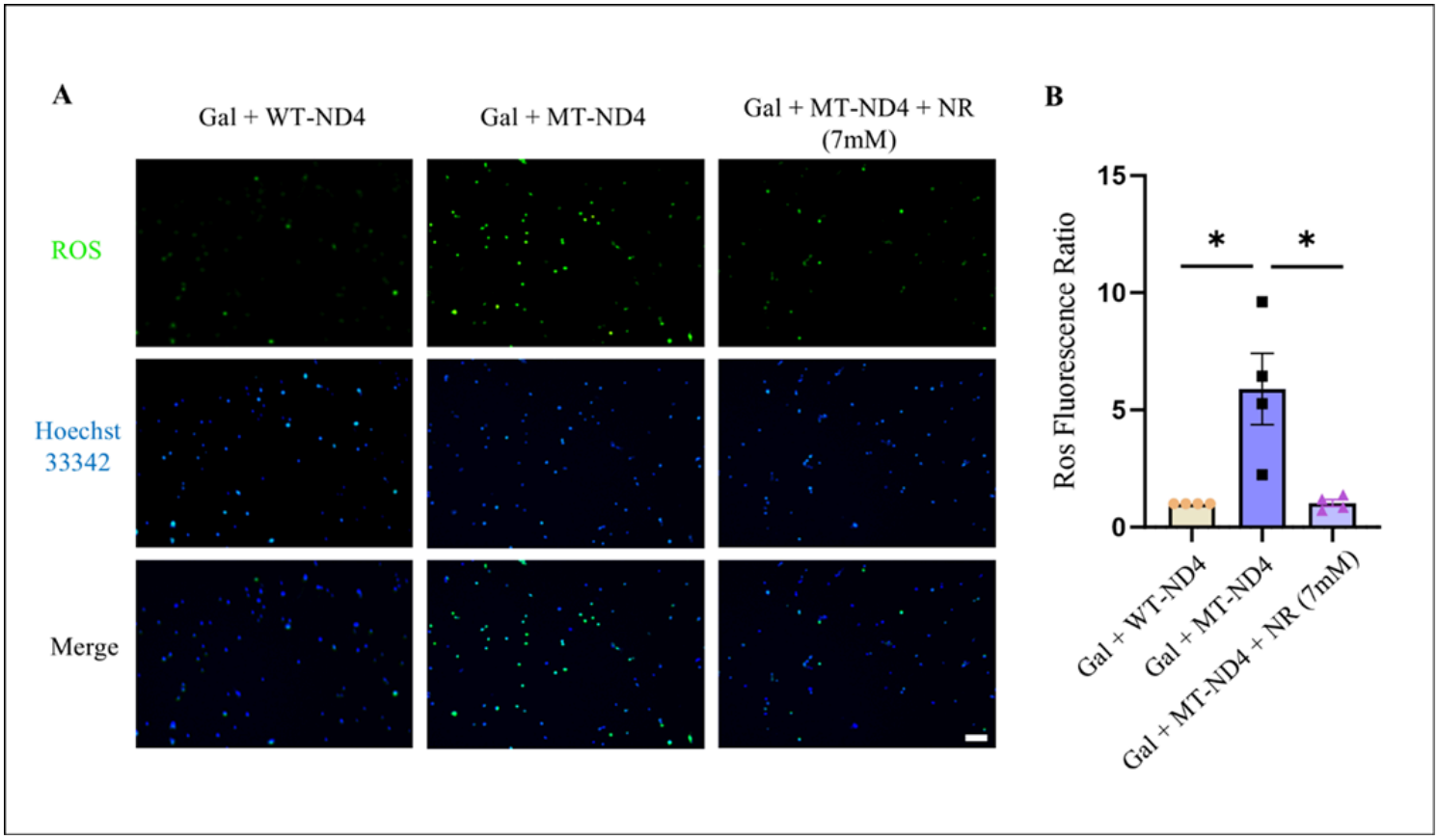

Figure 3

Influence of NR on mutant ND4 under oxidative stress. ROS levels were measured in 661W cells expressing exogenous ND4 cultured in galactose medium. Cells expressing the m.11778G>A mutation exhibited significantly increased ROS production compared to those expressing WT-ND4 under these conditions. However, treatment with NR effectively ameliorated this oxidative stress by reducing ROS levels. Scale bar = 100 μm. Data are presented as means ± SEM, n ≥ 4. Statistical significance was determined by one-way ANOVA. *$P < 0.05$.

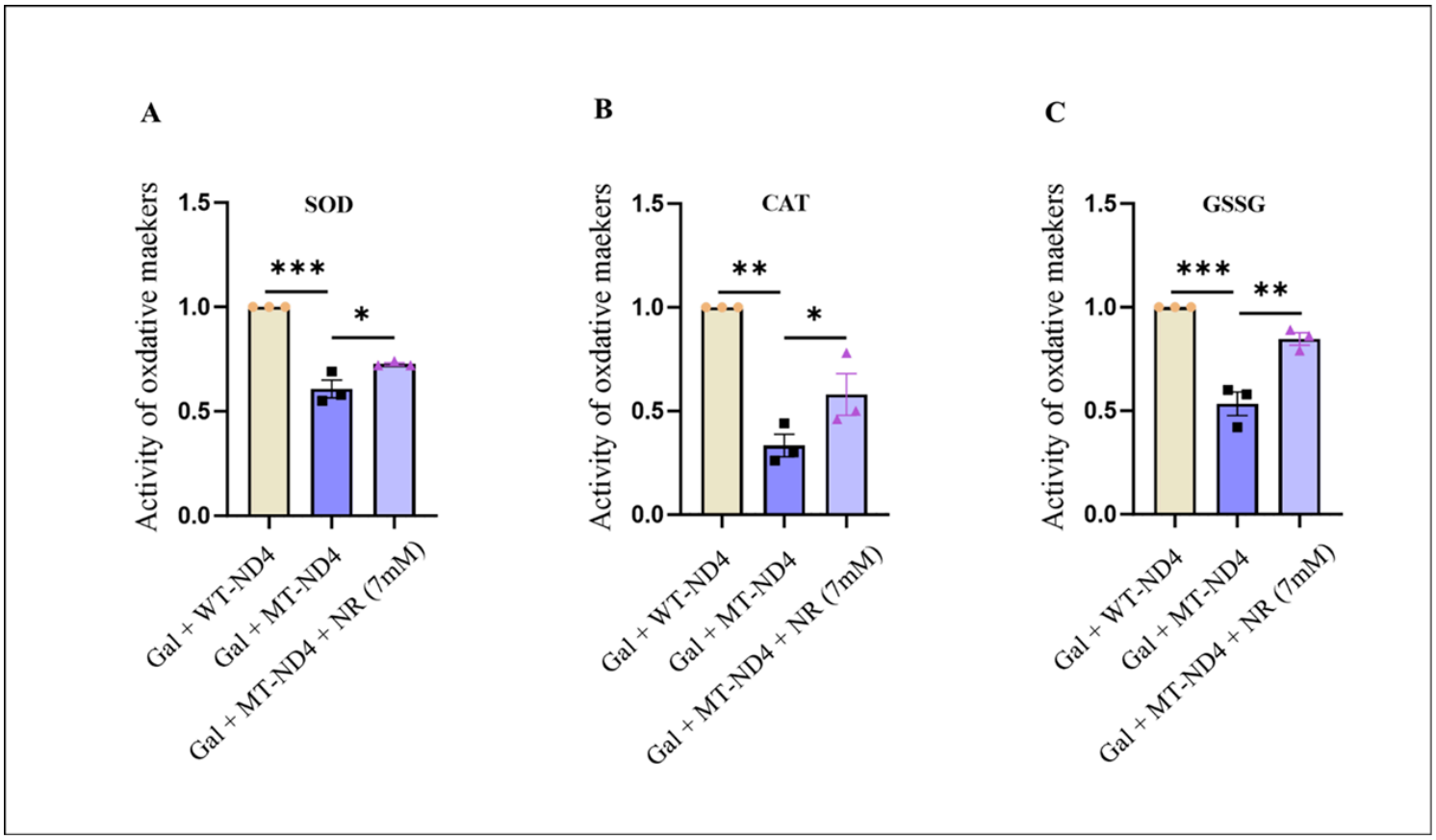

Figure 4

Influence of NR on mutant ND4 under antioxidative stress conditions. 661W cells expressing exogenous ND4 were cultured in galactose and free glucose DMEM. Levels of SOD (A), CAT (B), and GSSG (C) were measured. Cells expressing the m.11778G>A mutation in galactose medium showed decreased CAT, SOD, and GSSG levels, which were ameliorated by NR treatment. Data are presented as means ± SEM, n ≥ 4. Statistical significance was determined by one-way ANOVA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

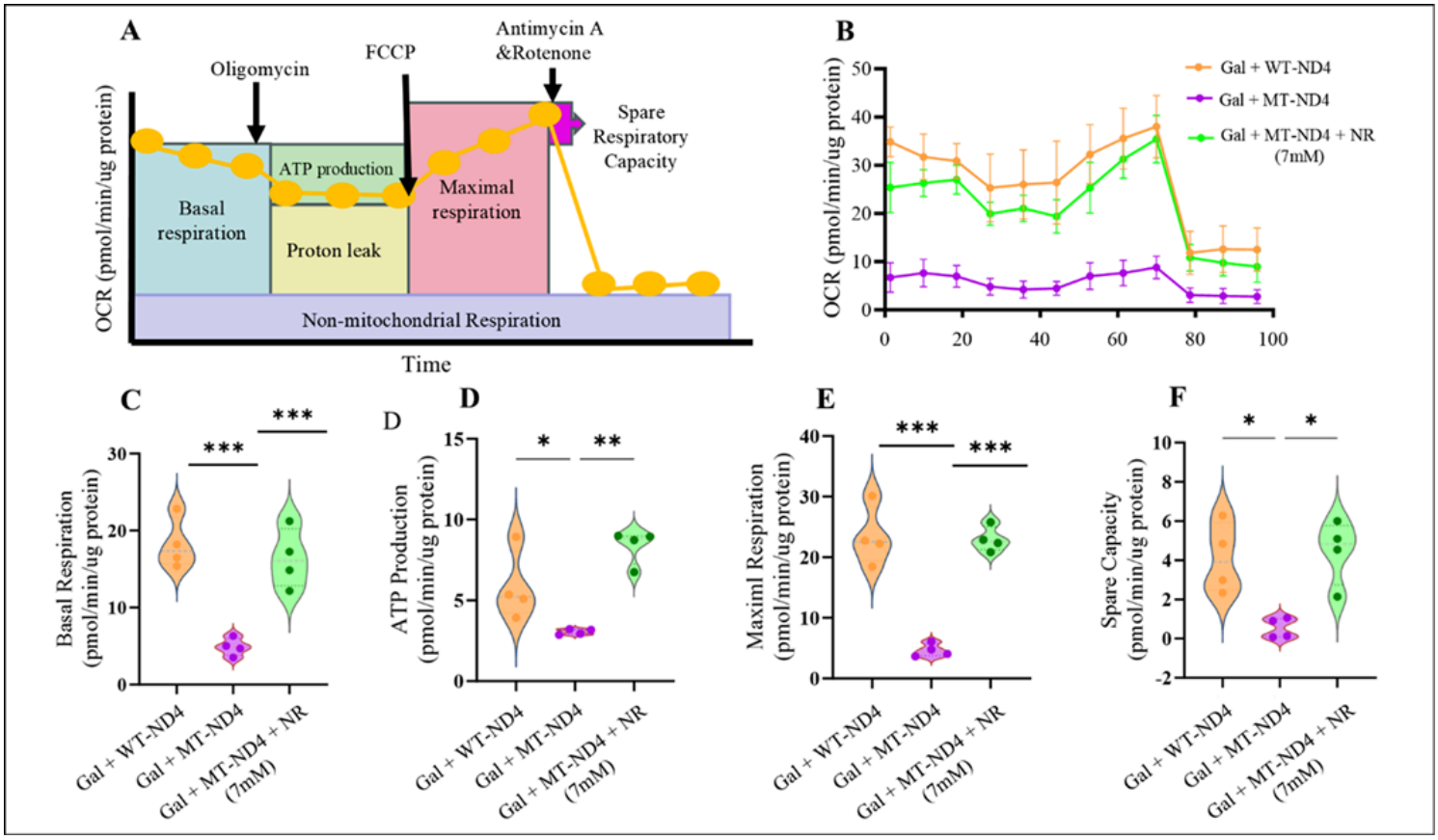

Figure 5

NR improved mitochondrial respiration. 661W cells expressing either wild-type or mutant exogenous ND4 genes were cultured in glucose-free, galactose-containing DMEM medium. Mitochondrial respiration parameters—including basal respiration, ATP-linked respiration, maximal respiration, and spare respiratory capacity—were assessed, demonstrating that NR treatment enhanced mitochondrial function in these cells under galactose conditions

(A and B) Representative OCR profiles: (A) schematic, (B) 661W cells; (C) basal OCR; (D) mitochondrial ATP production in different groups; (E) maximal respiration; (F) spare respiratory capacity. n = 4. Statistical significance was determined by one-way ANOVA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

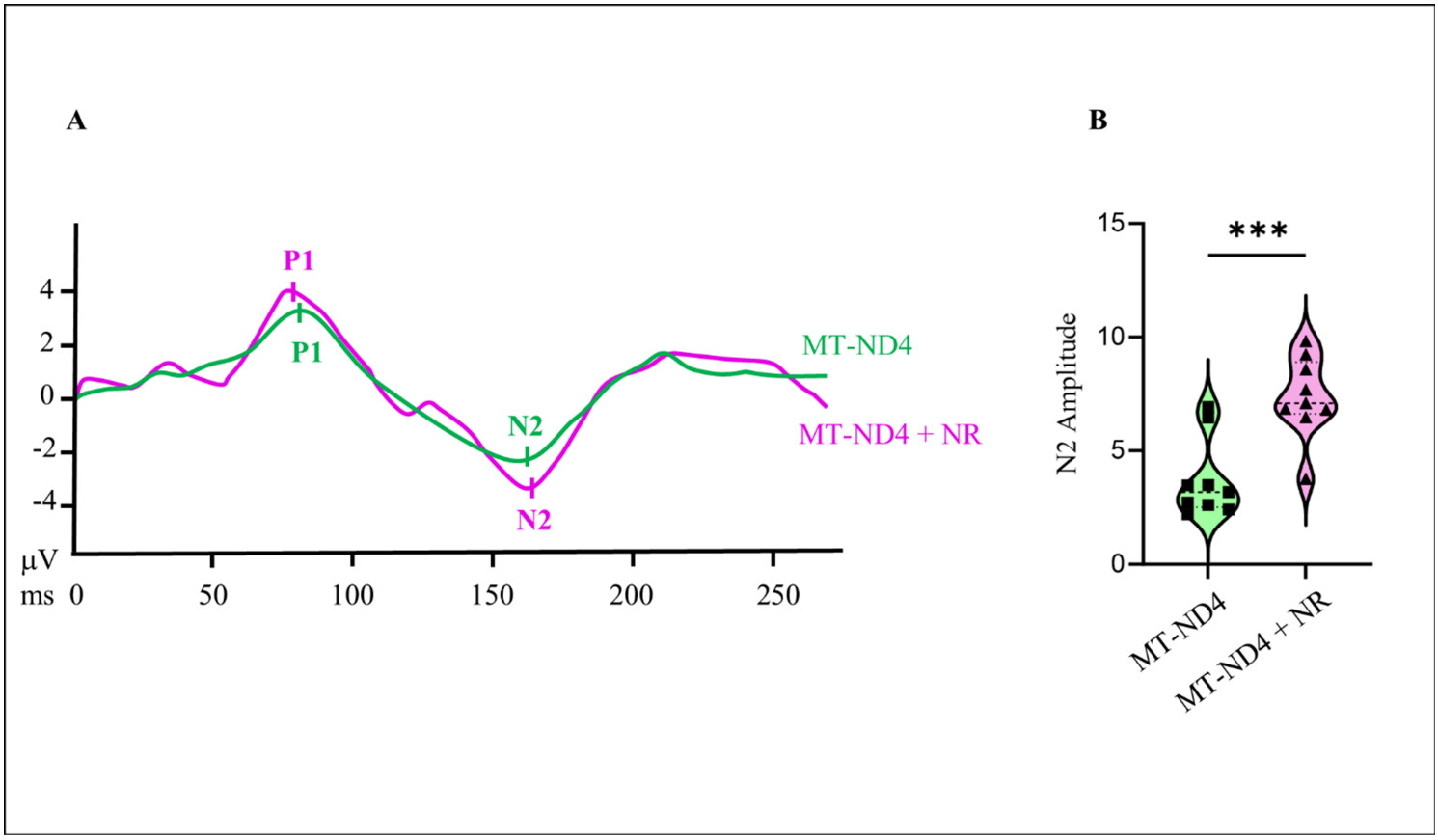

Figure 6

PERG tests in the assessment of RGC function. (A) PERG waveforms from the two treatment groups are displayed. (B) PERG recordings demonstrated a consistent increase in average amplitudes treated with both MT-ND4 and NR, compared to those receiving MT-ND4 treatment alone. This finding indicates a clear trend toward enhanced RGC function following NR administration, n = 9, ***$P < 0.001$.