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Report

Effects of Pyridoxine Hydrochloride on Mitochondrial Responses to Oxidative Stress in Human Dermal Fibroblasts

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Mitochondria play essential roles in cellular redox homeostasis and viability, and oxidative stress is known to impair mitochondrial function. In this study, we examined the effects of pyridoxine hydrochloride (vitamin B_6) on mitochondrial responses in normal human dermal fibroblasts (NHDFs) exposed to hydrogen peroxide (H_2O_2). Pre-treatment with pyridoxine hydrochloride was associated with changes in mitochondrial responses, including increased cell viability, higher PCR-based mtDNA amplifiability (an indirect indicator of mtDNA integrity), and maintenance of mitochondrial membrane potential under oxidative stress conditions. These observations indicate that pyridoxine hydrochloride influences mitochondrial responses to oxidative stress in this *in vitro* model, and suggest its utility for evaluating small-molecule antioxidants.

Key words pyridoxine hydrochloride, oxidative stress, mitochondrial function, mitochondrial DNA amplifiability, mitochondrial membrane potential, human dermal fibroblasts

INTRODUCTION

Mitochondria generate more than 90% of cellular energy through oxidative phosphorylation and play essential roles in maintaining metabolic and redox homeostasis.[1-3] Owing to its proximity to the respiratory chain and lack of histone protection, mtDNA is highly vulnerable to oxidative damage induced by reactive oxygen species (ROS).[4] In the context of skin biology, both chronological aging and photoaging have been associated with alterations in mtDNA, including increased frequencies of mtDNA deletions in sun-exposed or aged skin.[5, 6] These observations emphasize the importance of maintaining mitochondrial function under oxidative conditions.

To address mitochondrial dysfunction associated with oxidative stress, enzyme-based approaches such as photolyase and 8-oxoguanine DNA glycosylase (OGG1) have been investigated.[7, 8] While these enzymes participate in the processing of oxidative DNA lesions, their practical application is constrained by limitations related to structural stability, formulation, and intracellular delivery. Accordingly, attention has shifted toward small, chemically stable molecules with antioxidant properties, which may offer practical advantages in experimental and formulation settings. Several such small molecules, some of which are incorporated into cosmetic formulations, have been investigated for their potential to influence mitochondrial-related responses.

In this study, we focused on pyridoxine hydrochloride (vitamin B_6), a water-soluble and chemically stable small molecule, and evaluated its effects on mitochondrial responses under oxidative stress conditions. Using normal human dermal fibroblasts (NHDFs) exposed to hydrogen peroxide (H_2O_2), we examined whether pyridoxine hydrochloride is associated with changes in mitochondrial response parameters, including cell viability, PCR-based mtDNA amplifiability, and mitochondrial membrane potential. This study provides observational evidence regarding the effects of pyridoxine hydrochloride on mitochondrial responses to oxidative stress in an *in vitro* model.

MATERIALS AND METHODS

Reagents Mevalonolactone and niacinamide were obtained from Nikko Chemicals Co., Ltd. (Tokyo, Japan). Panthenol was prepared by BASF Japan Co., Ltd. (Tokyo, Japan). Pyridoxine hydrochloride was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Hydrogen Peroxide (H_2O_2) was purchased from Fujifilm Wako Pure Chemical Co., Ltd. (Osaka, Japan). The JC-1 MitoMP detection kit was obtained from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan).

Cell Culture NHDFs were purchased from Lonza Co., Ltd. (Tokyo, Japan) and maintained in FGM™-2 Fibroblast Growth Medium-2 BulletKit™ (Lonza Co., Ltd. Tokyo, Japan) according to the manufacturer's protocol. These cells are pri-

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mary human dermal fibroblasts, and cells between passages 4 and 8 were used for the experiments.

Cell Viability Assay NHDFs were seeded into a 96-well plate and incubated for 24 h in a 5% CO_2 incubator. The cells were treated with each compound as indicated. All compounds were prepared as stock solutions and diluted in culture medium to obtain the final concentrations indicated in Table 1. After 24 h, the cells were washed with PBS and then treated with H_2O_2 for 1 h. The concentration of H_2O_2 used in this study was selected based on preliminary experiments to produce a measurable reduction in cell viability while avoiding excessive cell death. Next, the cell viability assay was performed using Cell Counting Reagent SF (NACALAI TESQUE, Co., Ltd., Kyoto, Japan) according to the manufacturer's protocol. The absorbance values of the resultant formazans were measured at 450 nm using a Spectra Max iD3 microplate reader (Molecular Devices Japan, Co., Ltd., Tokyo, Japan).

qPCR-Based Assessment of mtDNA Amplifiability under Oxidative Stress NHDFs were seeded into a 24-well plate and incubated for 24 h in a 5% CO_2 incubator. The cells were treated with the compounds and H_2O_2 as described above for the cell viability assay. Total DNA was extracted using a Cica Geneus Total DNA Prep Kit (Kanto Chemical, Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Quantitative PCR (qPCR) analysis was conducted using THUNDERBIRD SYBR qPCR Mix (Toyobo, Co., Ltd., Osaka, Japan) and the Human Real-time PCR Mitochondrial DNA Damage Analysis Kit (Detroit R&D, Co., Ltd., Detroit, MI) according to the manufacturer's protocols. Relative mtDNA amplifiability was calculated using the ΔCt method and normalized to GAPDH as an internal reference gene.

Post-Stress mtDNA Amplifiability Recovery Assay NHDFs were seeded into a 24-well plate and incubated for 24 h in a 5% CO_2 incubator. The cells were treated with H_2O_2 for 45 min. After washing out, each compound was applied to the cells and incubated for 24 h. Similar to the descriptions above, total DNA was collected and PCR-based mtDNA amplifiability was analyzed by qPCR using the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, USA). Data were processed using QuantStudio™ Real-Time PCR Software (Applied Biosystems).

Analysis of Mitochondrial Membrane Potential NHDFs were seeded into a 96-well plate and incubated for 24 h in a 5% CO2 incubator. The cells were treated with the compounds and H_2O_2 as described above for the cell viability assay. Treated cells were incubated with JC-1 for 1 h at 37°C. Imaging buffer was added, then the cells were observed using ImageXpress Pico (Molecular Devices, USA). Fluorescence levels were detected using a SpectraMax iD3 multi-mode microplate reader (Molecular Devices), and the data were analyzed using SoftMax Pro 7.1 software (Molecular Devices).

The excitation/emission settings were standardized as:

Green (monomer): Ex 485 nm, Em 535 nm

Red (aggregate): Ex 535 nm, Em 595 nm

The JC-1 aggregate/monomer ratio was used as an indicator of mitochondrial membrane potential.

RNA Extraction, cDNA Generation, and qPCR NHDFs were seeded into a 24-well plate and incubated for 24 h in a 5% CO_2 incubator. The cells were treated with H_2O_2 for 45 min. After washing out, each compound was applied to the cells and incubated for 24 h. Total RNA was extracted using a FastGene RNA Basic Kit (NIPPON Genetics Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions, then cDNA was synthesized from 20 ng of total RNA using a FastGene Scriptase II cDNA synthesis 5x Ready Mix (Nippon Genetics Co. Ltd.). Next, qPCR analysis was conducted using THUNDERBIRD Next SYBR qPCR Mix according to the manufacturer's protocols. All primers used in this study are listed in Table 2.

Statistical Analysis Unpaired t-tests were used for comparisons between two groups. For multiple group comparisons, one-way ANOVA followed by the Bonferroni test was used to assess statistical significance. A P-value < 0.05 was considered statistically significant. Statistical analyses were performed using Microsoft Excel for t-tests and the Excel® Statistical Program File *ystat2018.xls* (Igakutoshoshuppan Ltd., Tokyo, Japan) for ANOVA and post-hoc testing. All experiments were independently repeated at least three times (n = 3), unless otherwise indicated.

RESULTS

To evaluate cellular responses under oxidative stress, NHDFs were pre-treated with four representative antioxidant-related compounds previously reported to influence cellular processes associated with aging[9-12] (Table 2) and subsequently exposed to H_2O_2. These compounds were selected because they are small molecules associated with antioxidant activity and cellular metabolic processes that have been reported to influence cellular responses related to oxidative stress and aging. H_2O_2 was used as a model oxidative stressor known to affect mitochondrial function. NHDFs were exposed to multiple concentrations of H_2O_2 following compound pre-treatment, and cell viability was assessed. As shown in Fig. 1, the addition of compounds A, B, C, and D (pyridoxine hydrochloride)

Table 1. Compounds Evaluated in This Study and Their Final Concentrations

ID	Materials	Addition concentration (% v/v)	Reference
A	Mevalonolactone	1	9
B	Niacinamide	0.1	10
C	Panthenol	1	11
D	Pyridoxine hydrochloride	0.05	12

Table 2. Primer Sequences Used for Quantitative PCR

Gene		Sequence
GAPDH	F	5′ -CATCCCTGCCTCTACTGGCGCTGCC- 3′
	R	5′ -CCAGGATGCCCTTGAGGGGGCCCTC- 3′
NTH1	F	5′ -AACAGGCTGAGGTGGACCAAGA- 3′
	R	5′ -CCAAGAGTCCATTGATCTCGTGC- 3′
OGG1	F	5′ -GGCTCAACTGTATCACCACTGG- 3′
	R	5′ -GGCGATGTTGTTGTTGGAGGAAC- 3′
NEIL1	F	5′ -CGGCGGCTGCGTGGAGAAGTC- 3′
	R	5′ -GTCCCAGCGGCCGAACCGGCG- 3′
NEIL2	F	5′ -GGGGCAGCAGTAAGAAGCTA- 3′
	R	5′ -GGAATAATTTCTTTCCATGGACCT- 3′
MUTYH	F	5′ -AACTCTTTGGCCCCTCTGTG- 3′
	R	5′ -GAAGGGAACACTGCTGTGAAG- 3′
APE1	F	5′ -CTGCTCTTGGAATGTGGATGGG- 3′
	R	5′ -TCCAGGCAGCTCCTGAAGTTCA- 3′
PNK	F	5′ -TTCGAGAGATGACGGACTCCTC- 3′
	R	5′ -AGGATGGCAGAGAAGCCTTCAG- 3′
POLG1	F	5′ -AAGCACTGTCTCGAACAGGG- 3′
	R	5′ -CACTGCAGCTCGCAAGTTCT- 3′

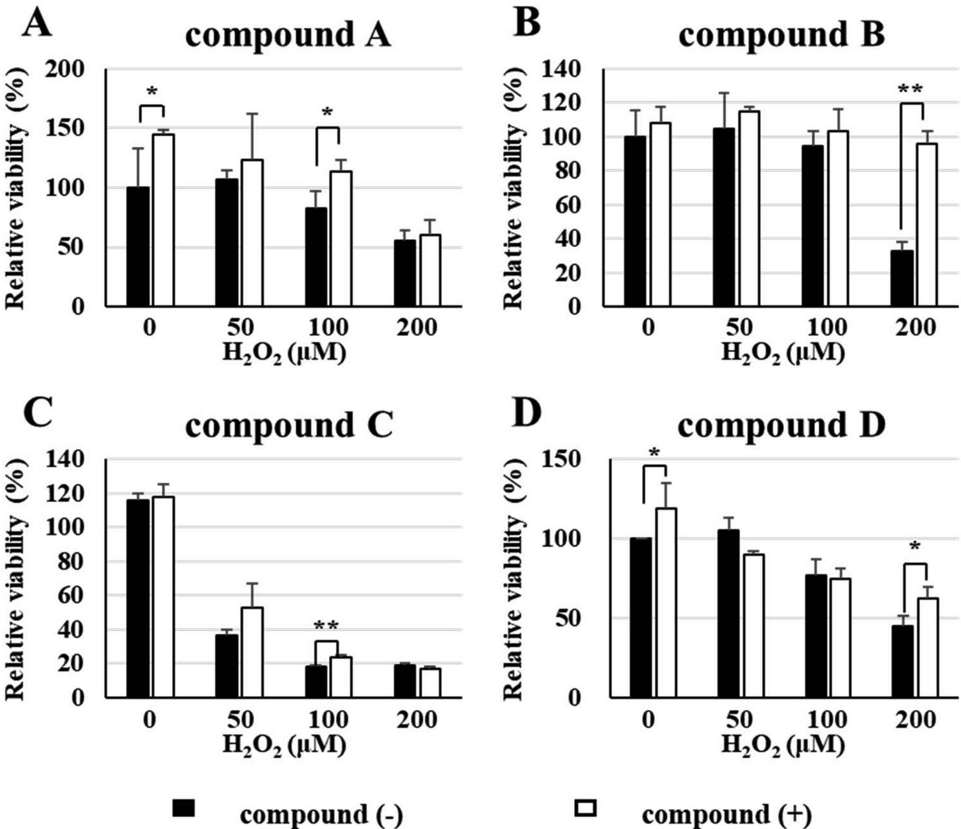


Fig. 1. Evaluation of Resistance to H_2O_2-induced Cytotoxicity

NHDFs were pre-treated with each compound (A to D) for 24 h and then treated with H_2O_2 (0, 50, 100, or 200 μM). Cell viability was assessed using Cell Counting Reagent SF 1 h later. A to D represent the material IDs in Table 2. The data are represented as the mean ± standard deviation (SD); n=3. Statistical analysis was performed using the unpaired t-test; *P<0.05, **P<0.01.

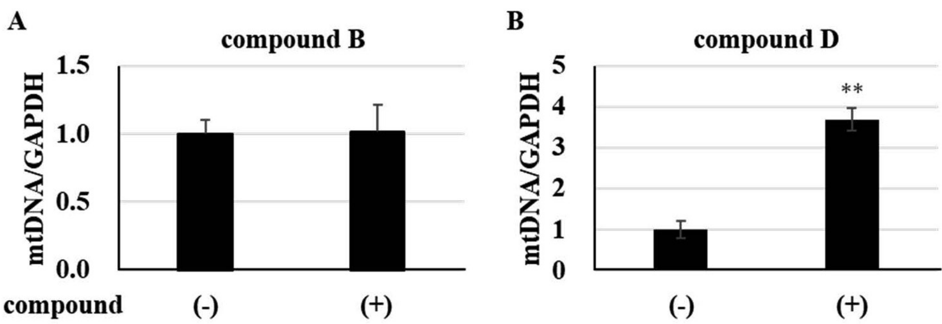


Fig. 2. Effects of Compounds on PCR-based mtDNA Amplifiability under H_2O_2 Exposure

NHDFs were incubated with each compound for 24 h, then stimulated with H_2O_2 (200 μM) for 1 h. Total DNA was extracted and relative mtDNA amplifiability was analyzed by qPCR. The data are represented as the mean ± SD; n=3. Statistical analysis was performed using the unpaired t-test; *P<0.05, **P<0.01.

was associated with attenuation of H_2O_2-induced cytotoxicity.

In the absence of H_2O_2 (0 μM), compounds A and D showed significantly higher cell viability compared with untreated control ($P < 0.05$). Under severe oxidative stress (200 μM H_2O_2), compounds B and D were associated with significant inhibition of cell death. Among the tested compounds, both B and D showed marked cytoprotective effects under this condition. For comparison, additional antioxidant substances, including plant-derived extracts, which were used as reference materials rather than chemically defined single compounds, were evaluated under comparable conditions (Table S1, Fig. S1). Taken together, although some additional test substances showed cytoprotective effects under oxidative stress conditions, none exhibited a cytoprotective profile clearly distinguishable from those observed for compounds B and D. Therefore, compounds B and D were selected for subsequent analyses focusing on mitochondrial response parameters.

All the compounds used in this study are known to have antioxidant capacity.[9-12] Accordingly, it remained unclear whether the attenuation of H_2O_2-induced cytotoxicity observed in Fig. 1 was attributable solely to antioxidant activity or was associated with changes in mitochondrial-related parameters. Because compounds B and D showed cytoprotective effects under severe oxidative stress (Fig. 1), we therefore examined whether H_2O_2-induced reductions in PCR-based mtDNA amplifiability (an indirect indicator of mtDNA integrity)

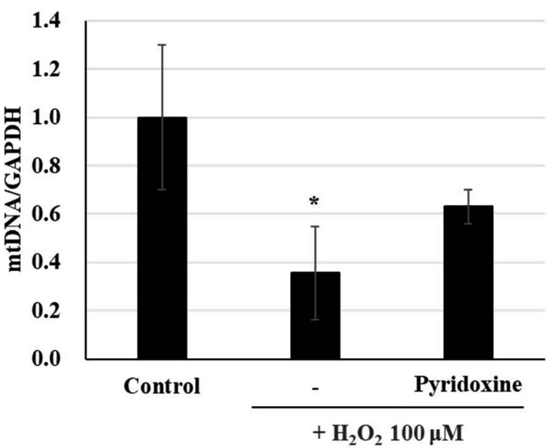
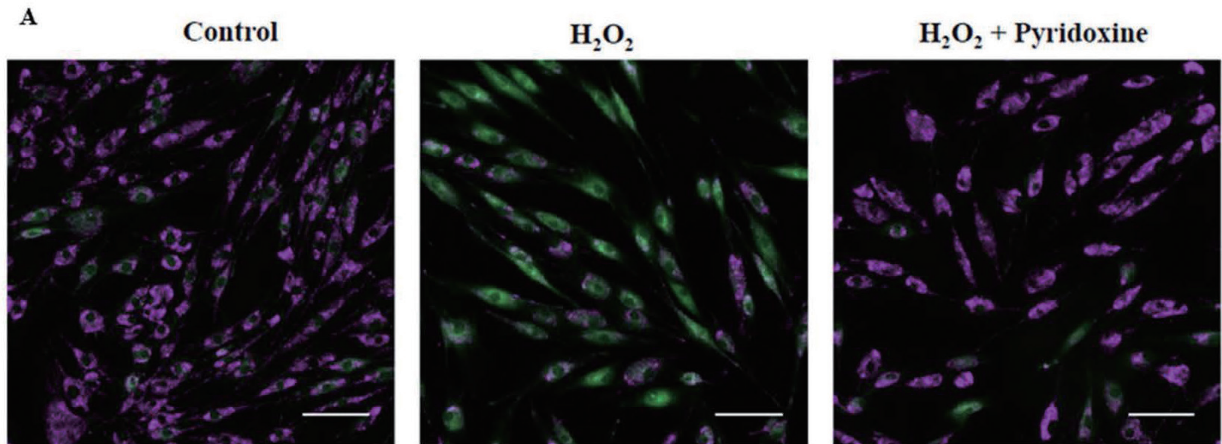


Fig. 3. Effects of Pyridoxine Hydrochloride on PCR-based mtDNA Amplifiability in a Post-stress Model

NHDFs were treated with H_2O_2 (100 µM) for 45 min, then treated with pyridoxine hydrochloride for 24 h. Total DNA was extracted and relative mtDNA amplifiability was analyzed by qPCR. The data are represented as the mean ± SD; n=3. Statistical analyses were performed by one-way ANOVA, followed by the Bonferroni test; **P<0.01 vs. control.

were differentially modulated by these compounds. NHDFs pre-treated with compounds B or D were exposed to 200 µM H_2O_2. As shown in Fig. 2, compound D (pyridoxine hydrochloride) was associated with maintenance of mtDNA amplifiability under H_2O_2 exposure, whereas compound B did not show a comparable association with mtDNA amplifiability under the same conditions. Based on this difference in mtDNA-associated responses, subsequent analyses focused on pyridoxine hydrochloride.

To further characterize mitochondrial responses following oxidative stress, we examined mtDNA amplifiability in a post-stress model using H_2O_2. NHDFs were first exposed to H_2O_2 to induce oxidative stress and subsequently treated with pyridoxine hydrochloride. As shown in Fig. 3, mtDNA amplifiability in pyridoxine hydrochloride–treated cells did not differ significantly from that observed in untreated control cells. Although no statistically significant difference was detected between the pyridoxine hydrochloride–treated group and the H_2O_2-treated group, mtDNA amplifiability values in the pyridoxine hydrochloride–treated group were intermediate between those of untreated control cells and H_2O_2-treated cells. These observations indicate that pyridoxine hydrochloride treatment was



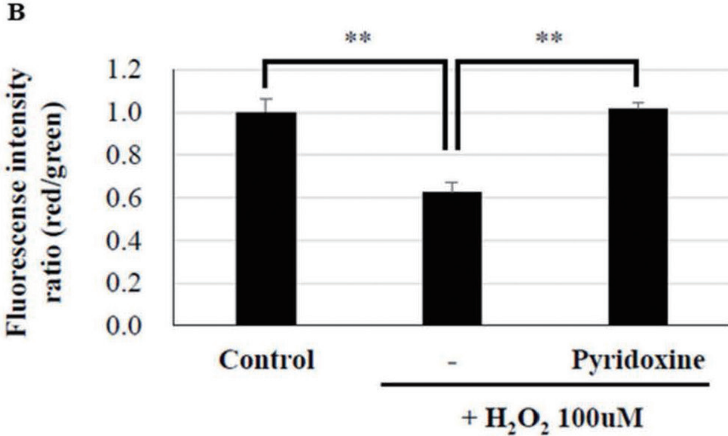


Fig. 4. Effects of Pyridoxine Hydrochloride on Mitochondrial Membrane Potential under Oxidative Stress

(A) NHDFs were treated with H_2O_2 (100 µM) for 45 min and then stimulated with solvent or pyridoxine hydrochloride (0.05%) for 24 h. Mitochondrial membrane potential was observed using an imaging device after JC-1 fluorescent staining. Scale bar: 100 µm; Magenta: JC-1 polymer; Green: JC-1 monomer. (B) Fluorescence intensity ratio of the mitochondrial membrane potential level (JC-1) with each treatment measured using a microplate reader. Green: Ex 485 nm, Em 525–545 nm; Red: Ex 535 nm, Em 585–605 nm. The data are represented as the mean ± SD; n=3. Statistical analyses were performed by one-way ANOVA, followed by the Bonferroni test; **P<0.01 vs. H_2O_2.

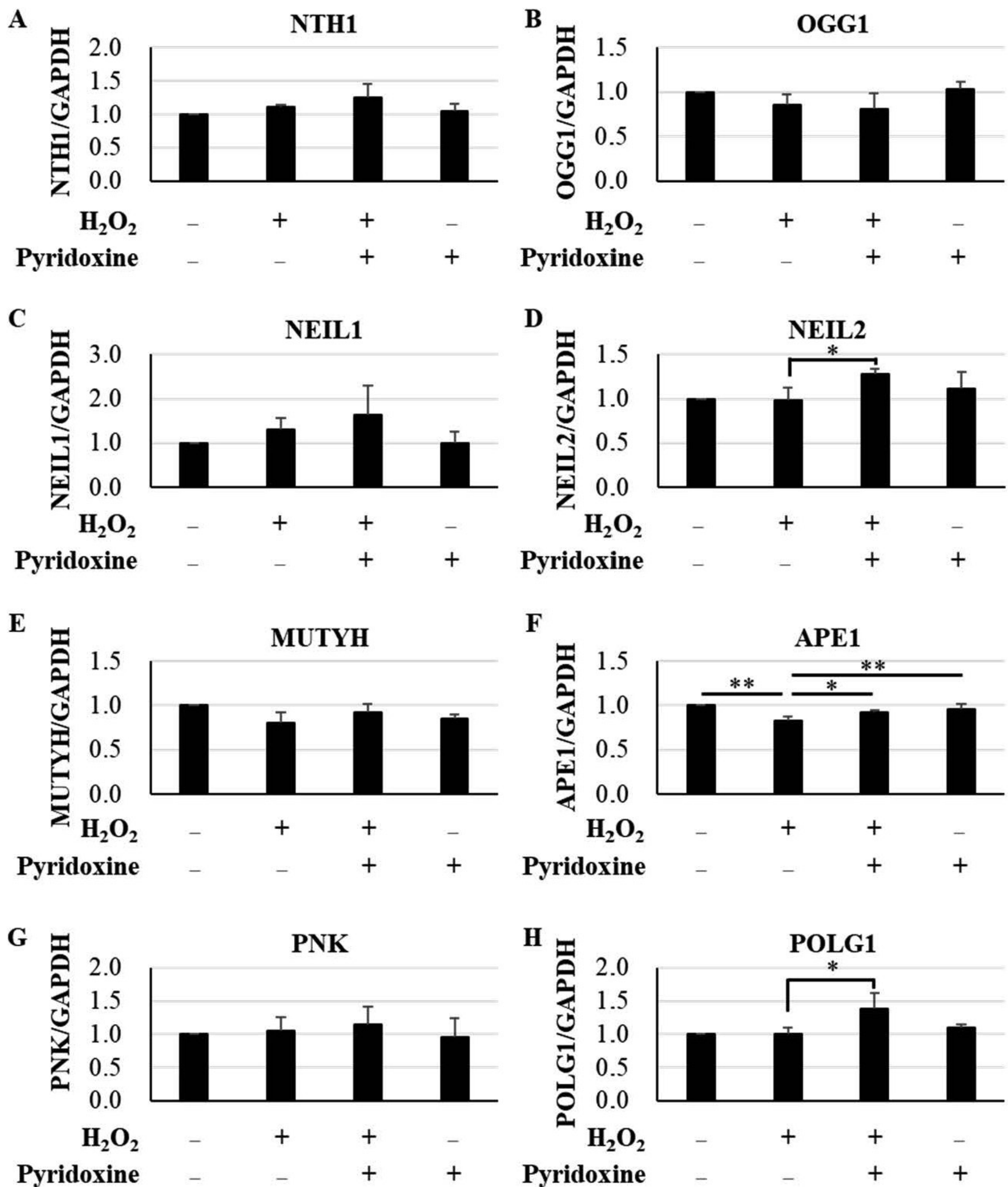


Fig. 5. Effects of Pyridoxine Hydrochloride on the Expression of Genes Associated with mtDNA Maintenance under Oxidative Stress

NHDFs were treated with H_2O_2 (100 µM) for 45 min, followed by incubation with culture medium (with or without 0.05% pyridoxine hydrochloride) for 24 h. Total RNA was then harvested. The relative mRNA expression levels of genes associated with mtDNA maintenance, (A) NTH1, (B) OGG1, (C) NEIL1, (D) NEIL2, (E) MUTYH, (F) APE1, (G) PNK, and (H) POLG1 were evaluated by qPCR and normalized to those of GAPDH. The data are represented as the mean ± SD; n=3. Statistical analyses were performed by one-way ANOVA, followed by the Bonferroni test; $^{*}P<0.05$, $^{**}P<0.01$ vs. H_2O_2.

associated with attenuation of H_2O_2-induced reductions in mtDNA amplifiability under post-stress conditions. To further examine mitochondrial functional responses under post-stress conditions, mitochondrial membrane potential and the expression of mtDNA maintenance-related genes were evaluated using the same experimental conditions as those used in Fig. 3.

Because mitochondrial membrane potential represents a functional parameter distinct from mtDNA-associated indicators, we next evaluated mitochondrial membrane potential under oxidative stress conditions. Normal mitochondria maintain a membrane potential that supports intracellular energy production, and oxidative stress is known to disrupt this parameter. NHDFs were exposed to H_2O_2 to induce oxidative stress and subsequently treated with pyridoxine hydrochloride.

As shown in Fig. 4A, representative JC-1 fluorescence images indicate that H_2O_2 treatment markedly reduced mitochondrial membrane potential, whereas cells treated with pyridoxine hydrochloride exhibited a higher magenta/green fluorescence signal compared with H_2O_2-treated cells, consistent with partial attenuation of H_2O_2-induced membrane potential reduction. Quantitative analysis (Fig. 4B) showed that mitochondrial membrane potential in pyridoxine hydrochloride–treated cells remained below that of untreated control cells but was higher than that observed following H_2O_2 treatment alone. Taken together, these results indicate that pyridoxine hydrochloride treatment was associated with attenuation of H_2O_2-induced reductions in mitochondrial membrane potential.

Because multiple enzymes involved in mtDNA maintenance are known to be encoded in the nuclear genome, we

next examined mRNA expression levels of selected mtDNA maintenance–related genes following pyridoxine hydrochloride treatment under oxidative stress conditions. NHDFs were first exposed to H_2O_2 and subsequently treated with pyridoxine hydrochloride. As shown in Fig. 5, pyridoxine hydrochloride treatment was associated with increased mRNA expression levels of nei-like DNA glycosylase 2 (NEIL2), apurinic/apyrimidinic endonuclease 1 (APE1), and DNA polymerase gamma subunit 1 (POLG1). No consistent changes were observed for the other genes examined under these conditions. These results indicate that pyridoxine hydrochloride treatment is accompanied by changes in mRNA expression of selected genes related to mtDNA maintenance under oxidative stress.

DISCUSSION

In the present study, we evaluated mitochondrial responses to oxidative stress using an H_2O_2-induced model in NHDFs. The experimental design included both a pre-stress model, in which cells were treated with the compound prior to H_2O_2 exposure (Fig. 2), and a post-stress model, in which compound treatment was applied after oxidative stress induction (Fig. 3). The analysis was conducted from three perspectives: (1) cytoprotective effects under oxidative stress (Fig. 1), (2) changes in PCR-based mtDNA amplifiability (Fig. 2), and (3) post-stress alterations in mtDNA amplifiability (Fig. 3). Under severe oxidative stress, compounds B and D exhibited comparable cytoprotective effects. However, only pyridoxine hydrochloride was associated with maintenance of mtDNA amplifiability under the same conditions. In the post-stress model, mtDNA amplifiability in pyridoxine hydrochloride–treated cells remained at an intermediate level between untreated controls and H_2O_2-treated cells, without statistically significant restoration. These observations suggest that pyridoxine hydrochloride may be associated with a maintenance-oriented mitochondrial response rather than direct mtDNA repair.

Additional analyses using other antioxidant-related substances provided further context for these findings. Although some substances demonstrated cytoprotective effects under H_2O_2 exposure and were associated with relatively higher mtDNA amplifiability (Fig. S2), others exhibited cytoprotection without corresponding changes in mtDNA amplifiability in the post-stress model (Fig. S3). These findings indicate that suppression of cell death or general antioxidant capacity alone may not fully account for changes in mtDNA-associated responses under oxidative stress.

Consistent with the mtDNA-related observations, pyridoxine hydrochloride was also associated with attenuation of H_2O_2-induced reductions in mitochondrial membrane potential (Fig. 4). Although membrane potential did not return to control levels, the reduction observed following oxidative stress was partially mitigated. While direct chemical interaction with H_2O_2 cannot be completely excluded, the observed differences in mtDNA-related responses and mitochondrial membrane potential suggest that the effects may not be fully explained by simple antioxidant scavenging alone. Furthermore, pyridoxine hydrochloride treatment was accompanied by altered mRNA expression of selected genes related to mtDNA maintenance, including NEIL2, APE1, and POLG1 (Fig. 5). It should be noted that these analyses were limited to mRNA expression levels, and the functional implications at the protein or enzymatic level remain to be clarified. The present study focused on describing mitochondrial responses associated with pyridoxine hydrochloride under oxidative stress conditions rather than elucidating the detailed molecular mechanisms.

Accumulation of mtDNA alterations during aging has been associated with mitochondrial dysfunction, increased reactive oxygen species production, and loss of membrane potential. In this context, the present findings suggest that pyridoxine hydrochloride may be associated with modulation of mitochondrial responses under oxidative stress conditions *in vitro*. However, the evaluation of mtDNA was based on PCR-derived amplifiability as an indirect indicator, and direct assessment of DNA damage or repair was not performed. Further studies, including protein-level analyses, additional cell types, *in vivo* models, and alternative stress conditions, will be necessary to clarify the biological significance of these observations.

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Conflict of Interest Hideki Takahashi is an employee of ADJUVANT COSME JAPAN Co., Ltd. Kenji Masuda is an employee of ADJUVANT COSME JAPAN Co., Ltd. Yuma Yamada received a research grant from ADJUVANT COSME JAPAN Co., Ltd. and serves as a consultant to ADJUVANT COSME JAPAN Co., Ltd.

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