Improved Effect of a Mitochondria-Targeted Antioxidant on Hydrogen Peroxide-Induced Oxidative Stress in Human Retinal Pigment Epithelium Cells

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Abstract

Background: Oxidative damage in retinal pigmented epithelium (RPE) cells contributes to the development of age-related macular degeneration, which is among the leading causes of visual loss in elderly people. In the present study, we evaluated the protective role of TPP-Niacin against the hydrogen peroxide (H_2O_2)-induced oxidative stress to RPE cells.

Methods: The cellular viability, lactate dehydrogenase, reactive oxygen species (ROS), and mitochondrial function were determined in the retinal ARPE-19 cells under the treatment with H_2O_2 or pre-treatment with TPP-Niacin. The expression level of mitochondrial related genes and some transcription factors were assessed using real-time polymerase chain reaction (RT-PCR).

Results: TPP-Niacin significantly improved cell viability reduction, reduced ROS generation and increased the antioxidant enzymes in H_2O_2-treated ARPE-19 cells. Mitochondrial dysfunction from H_2O_2-induced oxidative stress was also significantly diminished by the TPP-Niacin treatment, reduced generation of ROS, an ameliorated reduction of mitochondrial membrane potential (MMP) and an upregulated mitochondrial associated gene. In addition, TPP-Niacin markedly enhanced the expression of transcription factors (PGC-1α and NRF2) and antioxidant associated genes (especially, HO-1 and NQO-1).

Conclusion: We proved the protective effect of TPP-Niacin against H_2O_2-induced oxidative stress in RPE cells. TPP-Niacin is believed to have played a protective role against mitochondrial dysfunction by up-regulating antioxidant-related genes such as PGC-1α, NRF2, HO-1 and NQO-1 in RPE cells.

Introduction

The Age-related macular degeneration (AMD) is one of the most common causes of irreversible blindness in the elderly population in developed countries. There are two major forms of AMD: the non-neovascular dry form of AMD affecting approximately 85–90% of patients and the neovascular exudative wet form affecting the remaining 10–15% of patients [1, 2]. The dry AMD (atrophy AMD) originates from changes in the pigmentation of the retinal pigmented epithelial (RPE) cells and sub-retinal deposits due to lipid and protein accumulation between the RPE cells and Bruch's membrane, a condition termed as drusen. These processes finally result in RPE cell death, photoreceptor dysfunction, and loss of vision [1–3]. At present, the treatment of anti-vascular endothelial growth factor therapy (anti-VEGF therapy) has led to extraordinary improvements in wet AMD; however, effective treatment for dry AMD is unfortunately not yet available [4, 5]

Despite being a condition of unknown etiology, oxidative stress has been considered as a major influence on RPE cells in AMD pathophysiology [5–7]. RPE cells have high metabolic rates with enriched mitochondrial population, and the oxidative phosphorylation process produces the cellular energy source adenosine triphosphate (ATP), indicating the making of high amounts of reactive oxygen species (ROS) [8]. Thus, RPE cells exist in an environment of abundant endogenous ROS, while long-term accumulation

of such oxidative damage leads to dysfunctions in the RPE cells and raises their susceptibility to oxidative stress. Meanwhile, the ROS predominantly target the mitochondria, destroying their membrane integrity, causing dissipations of the mitochondrial membrane potential (ΔΨm, MMP), and resulting in mitochondrial dysfunction as well as governing cell survival. Indeed, intramitochondrial oxidative stress is connected with processes ruling cell survival, such as mitochondrial flexibility, apoptosis, and autophagy in AMD [9]. Therefore, defending the RPE cells from oxidative damage is considered to be a noteworthy option for preventing the occurrence or weakening of AMD.

To improve the therapeutic effects and diminish the side effects of the chemicals, a number of researchers have investigated strategies for subcellular targeting, especially mitochondria-targeting. The selective targeting of antioxidants toward mitochondria by covalent conjugation to the lipophilic triphenylphosphonium (TPP) cation is a widely used method [10–12]. TPP, which is a well-known mitochondrial targeting moiety, is a membrane-permeant lipophilic cation that is quickly gathered by a factor of several folds within the mitochondria in vivo and is controlled by the large MMP [13]. Since the development of mitochondrial target compounds, there have been several reported mitochondria-targeted antioxidants, including MitoQ [14], MitoC [15], MitoE [16], and TPP-IOA [17]. Most of these studies employed traditional antioxidants such as vitamin C, vitamin E, and oleic acid to obtain TPP-conjugates. Among these vitamins, vitamin B3 (niacin or nicotinic acid) is widely recognized as a potent antioxidant that also exerts powerful lipid-lowering effects with high concentration [18–21]. However, according to our literature search, no one has been shown that not only the synthesis of TPP-conjugated niacin (TPP-Niacin) for mitochondrial-targeting but also the antioxidant effect of TPP conjugated niacin. Therefore, in this study, we synthesized TPP conjugated niacin and investigated whether TPP-Niacin could protect RPE cells from hydrogen peroxide (H_2O_2)-induced damage. We also evaluated the molecular actions underlying the effects of TPP-niacin on H_2O_2 stimulated ARPE-19. In brief, we confirmed that TPP-Niacin exerted a protective role against H_2O_2-induced cytotoxicity and mitochondrial dysfunction via upregulated the antioxidant-associated genes in RPE cells.

Methods

Materials and Reagents

The human retinal pigment epithelial ARPE-19 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 media (DMEM/F12), fetal bovine serum (FBS), penicillin/streptomycin, 2,7-dichlorofluorescein diacetate (H_2DCF-DA) were purchased from Thermo Fisher Scientific (Wilmington, DE, USA). Hydrogen peroxide, dihydroethidium (DHE), JC-10 assay kit, N-acetyl-cysteine (NAC) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were purchased from Sigma-Aldrich (St. Louis, MO, USA, owned by Merck KGA). TPP-Niacin was chemically synthesized with reference to a previous report and patent application, and Niacin was used as reference control [12, 14, 22, 23]. Cell Counting Kit-8 (CCK-8) and Lactate dehydrogenase (LDH) assay were purchased from Dojindo Molecular Technologies (Japan). The kits used to determine

the superoxide dismutase 1 and 2 (SOD1 and 2), catalase and Glutathione peroxidase activities, Tetramethylrhodamine, ethyl ester (TMRE) reagent were obtained from Abcam (UK).

Cell Culture

The human retinal pigment epithelia ARPE-19 cells were routinely maintained in DMEM/F12 (Thermo Fisher Scientific) supplemented with 10% FBS and 1% penicillin/streptomycin (Thermo Fisher Scientific. The cells were incubated at 37°C in an atmosphere conditioning 5% CO_2.

Cell Viability Assay

The ARPE-19 cells were plated in 96-well plates of $5x10^3$ cells per well and incubated for 24 h. The next day, the cells were exposed to different concentrations of TPP-Niacin for 24, 48, and 72 h. The cell viability was assessed using Cell Counting Kit-8 (CCK) assay (Dojindo Molecular Technologies). In brief, 10 µL of the CCK reagent was added and incubated for one hour. Then, absorbance was measured at 450 nm using a microplate reader (Bio-Tek, Winooski, VT, USA). To evaluate suitable H_2O_2 concentration for oxidative damage and cytotoxic induction, the cells were seeded in 96-well plates for 24 h and incubated with variouse concentrations of hydrogen peroxide (H_2O_2, Sigma-Aldrich) for another 24 h. Then, cell viability was evaluated by CCK-8 by the same method as before. To examine the protective effect of TPP-Niacin against H_2O_2-induced oxidative damage, cells were seeded in 96-well plates and pre-treated with different concentrations of TPP-Niacin for 2 h, and followed by exposure to 300µM H_2O_2, for an additional 24 h. Then, cell viability was evaluated by CCK-8 as aforementioned. All samples were run in triplicates, and each experiment was repeated three times.

Lactate Dehydrogenase (LDH) Release Assay

Cell cytotoxicity was determined by LDH activity released from damaged cells using the Cytotoxicity LDH assay Kit (Dojindo Molecular Technologies). In brief, ARPE-19 cells were plated in 96-well plates (5000 cells/well) and pre-treated with different concentrations of TPP-Niacin for 2 h followed by exposure to H_2O_2 (300 µM) for 24 h. The background of LDH in the growth medium was measured and subtracted from all test samples. Basal levels of LDH (zero percentage of cell death), as measured in the supernatant of vehicle-treated cells, and maximal levels of LDH (cell death of one hundred percentage), measured by induction of lysis buffer for complete cell death, were averaged and used to calculate the percentage cell death using the manufacturer's protocol.

Measurement of ROS

The generation of intracellular ROS was examined using the ROS Detection Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, cells were grown in a 96-well black plate and subjected to different treatments with/without TPP-Niacin and H_2O_2. Then, the cells were incubated with 5 µM 2,7-dichlorofluorescein diacetate (H_2DCF-DA) or 10 µM dihydroethidium (DHE) at 37 °C for 20 min. The fluorescence intensity was measured using the fluorescence plate reader (Bio-Tek) at Ex./ Em. =

495/527 nm for H_2DCF-DA and Ex./ Em. = 535/610 nm for DHE. And then, DCFDA stained cell images were obtained with a fluorescent microscope IX51 with DP Controller (Olympus Optical, Japan). All samples were run in triplicates, and each experiment was repeated three times.

Antioxidant Enzyme Activity

ARPE-19 cells (2×10^6 cells per well) were plated onto 100 mm cell culture dishes and treated in the same steps as above. ARPE-19 cells were collected into the clean tubes with 100 µL Pro-Prep (Intron, Korea) lysis buffer for 20 min in ice after washing with PBS. Then, the supernatant was carefully collected after centrifugation and then protein concentration was calculated with a Bicinchoninic acid protein assay kit (BCA, Thermo Fisher Scientific). Intracellular activities of SOD1, SOD2, CAT, and GPx were detected with the commercial kits (Abcam, UK) following their directions. The results were shown as the percentage of the untreated group.

MMP and Staining

The MMP assay was conducted by following the manufacturer's instruction of the JC-10 MMP assay kit (Sigma-Aldrich). In brief, ARPE-19 cells (5×10^3 cells/well) seeded in a 96-well clear bottom black plate (Eppendorf Ltd., Germany) were treated with in the same steps as above. After 24 h, the JC-10 assay solution was added to the cells. And then, the plate was incubated under the dark condition for 30 min. Following the incubation, assay buffer B was added and the fluorescent intensity was measured at 490/525 nm and 540/590 nm using a multimode plate reader (Bio-Tek). The ratio of red/green fluorescent intensity was used to determine the MMP. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and N-acetyl-cysteine (NAC) were used as positive and antioxidant control, respectively. All samples were run in triplicates, and each experiment was repeated three times. To visualize the effect of TPP-Niacin on MMP, cells were stained with Tetramethylrhodamine, ethyl ester (TMRE) reagent (Abcam, UK). The stained cells were analyzed in an inverted fluorescent microscope IX51 with DP Controller (Olympus Corp., Japan) at Ex./ Em. = 549/575 nm.

Transmission Electron Microscopy

ARPE-19 cells (5×10^6 cells/well) seeded in 100 mm dishes and treated in the same steps as above. Cells were washed three times with 0.1 M Phosphate Buffer (PB) and fixed overnight in 3% glutaraldehyde at 4 °C and 1% osmium tetroxide solution as used for additional fixation for 30 min. Cells were dehydrated with a graded series of ethanol, and then dehydrated sample was embedded in epoxy Resin. The embedded sample was cut into ultrathin sections around 60 nm through an ultramicrotome (RMC MT-XL; RMC Products, Tucson, AZ, USA), and a transmission electron microscope (Hitachi H-7100, Japan) was used to observe.

RNA Collection and Quantitative PCR

Total RNA was collected using Trizol reagent (Thermo Fisher Scientific) and resuspended in RNAs free water. The concentration of each sample was determined using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with 1µg of RNA to produce the complimentary DNA (cDNA) using SensiFast cDNA synthesis kit (Bioline, London, UK). To measure the gene expression, a quantitative polymerase chain reaction (qPCR) was performed using 3µl of cDNA template and the Power SYBR Green Master Mix (Thermo Fisher Scientific) on the StepOnePlus (Applied system, USA). All samples were run in triplicates, and each experiment was repeated three times. Data was normalized to the mean expression of the housekeeping gene using GAPDH and quantified using the $2^{-\Delta\Delta}$CT method. The primer sequences used are inventoried in Table 1.

Statistics

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). All data were presented as mean±SD. Student's t-test was used to calculate statistical significance between 2 groups. Statistical significance is denoted by the following: ns P >0 05, ∗P <0 05, ∗∗P <0 01, and ∗∗∗P <0 001.

Results

Cell Viability and Protective Effect of TPP-Niacin on ARPE-19 Cells

To evaluate the optimal concentration of TPP-Niacin to be used without causing cytotoxicity, ARPE-19 cells were incubated with various concentrations of TPP-Niacin for 24, 48, and 72 h. As shown in Figure 1B, TPP-Niacin at 25 and 50 µM concentrations did not show any cytotoxicity in the ARPE-19 cells compared to the control group, whereas concentrations between 100 and 400 µM of TPP-Niacin attenuated the cell viability at 48 and 72 h. Thus, 25 and 50 µM of TPP-Niacin were used in the following experiments. To determine a suitable H_2O_2 concentration for oxidative damage, the cells were exposed to various concentrations of H_2O_2 for 24 h. The H_2O_2 treatment (around 300 µM) significantly reduced cell viability, resulting in 46.3 % cell death (Figure 1C). Therefore, the H_2O_2 concentration of 300 µM was utilized in the subsequent experiments. To test the defensive effect of TPP-Niacin on H_2O_2-induced cell death, the cells were treated with TPP-Niacin for 2 h before being exposed to H_2O_2 for 24 h. As shown in Figure 1D, the pre-treatment with 25 and 50 µM of TPP-Niacin significantly improved H_2O_2-induced reduction of ARPE-19 cells (at 25 µM: 70 ± 6.1%; at 50 µM: 72 ± 7.5%). The protective effect of TPP-Niacin was also assessed by the lactate dehydrogenase (LDH) assay. The TPP-Niacin cells pre-treated for 2 h significantly reduced H_2O_2-induced LDH level (Figure 1E). In addition, to compare the protective effects of niacin and TPP-Niacin, the cell viability assay was performed with and without oxidative stress. The test compounds did not show any cytotoxicity, and the pre-treatment of cells with niacin, TPP-Niacin, and N-acetyl-cysteine (NAC) for 2 h significantly protected the cells from H_2O_2-induced cell death (Supplementary Figures 1A and 1B).

TPP-Niacin Suppressed H_2O_2-Induced Oxidative Stress in ARPE-19 Cells

The excessive accumulation of ROS is regarded to be one of the main sources of cell damage induced by H_2O_2. Intracellular ROS signals were quantified using a fluorescence probe, 2,7-dichlorofluorescein diacetate (DCFDA) and dihydroethidium (DHE) reagent, in the ARPE-19 cells. As shown in Figure 2, compared with the control group, 300 μM H_2O_2 caused a significant increase in the fluorescent intensity of DCFDA (A) and DHE (B) in the ARPE-19 cells. However, pre-treatment with TPP-Niacin in the ARPE-19 cells markedly decreased the ROS levels when compared to H_2O_2 treatment alone (Figures 2A and 2B). The suppressive activity of TPP-Niacin was also observed in the fluorescence image, as illustrated in Figure 2C. Meanwhile, to find the role of the antioxidant enzymes in the protective effect due to TPP-Niacin against oxidative damage, the expressions of major antioxidant enzymes, including SOD1, SOD2, catalase (CAT), and GPx were measured by ELISA. The pre-treatment with TPP-Niacin effectively replenished the activities of SOD1 and SOD2 in the ARPE-19 cells, which were earlier decreased by treatment with 300 μM H_2O_2 (Figures 3A and 3B). Additionally, the activity of CAT was significantly improved by pre-treatment with TPP-Niacin compared to treatment with H_2O_2 alone (Figure 3C). As shown in Figure 3D, pre-treatment with TPP-Niacin markedly enhanced the GPx level, which was almost abrogated by treatment with H_2O_2. In addition, the ARPE-19 cells pre-treated with niacin and TPP-Niacin showed a marked reduction in H_2O_2-induced ROS production. As expected, TPP-Niacin exerted a somewhat higher protective effect against oxidative stress compared to niacin-treated cells (as shown in Supplementary Figures 2A and 2B).

TPP-Niacin Decreased H_2O_2-Induced Change of MMP and Mitochondrial Morphology

Mitochondrial dysfunction causes loss of MMP ($\triangle\Psi m$). To determine whether TPP-Niacin could decrease H_2O_2-induced $\triangle\Psi m$ loss, the $\triangle\Psi m$ of the ARPE-19 cells was performed by analyzing the red/green fluorescence intensity ratio via the JC-10 assay. the ARPE-19 cells were exposed to 300 μM H_2O_2 and resulted in a decrease of red/green fluorescence intensity ratio indicating $\triangle\Psi m$ dissipation, similar to the carbonyl cyanide m-chlorophenyl hydrazone (CCCP; mitochondrial oxidative phosphorylation uncoupler) group. However, pre-treatment with TPP-Niacin at 25 and 50 μM for 2 h improved the H_2O_2-induced $\triangle\Psi m$ loss to the same extent as the antioxidant positive control (NAC), as shown in Figure 4A. The same tendency was observed in ARPE-19 cells pre-treated with TPP-Niacin, when compared with those treated with H_2O_2 alone, using tetramethylrhodamine ethyl ester (TMRE) reagent for visual illumination of the mitochondria (Figure 4B). Interestingly, pre-treatment with the parent compound (niacin) for 2 h did not show any improvement in the H_2O_2-induced $\triangle\Psi m$ loss (Supplementary Figure 3); thus, the results revealed that TPP-Niacin could successfully be used as a target for mitochondria. Additionally, mitochondrial morphology was characterized by electron microscopy (EM) (Figure 4C). The H_2O_2 treated cells showed less dense cytoplasm and larger numbers of swollen mitochondria with disrupted cristae, whereas these appeared rather uniform, including intact cristae of the mitochondria morphology, in the control group. Although some mitochondria presented a disorganized structure with disturbed cristae and swollen appearance in the TPP-Niacin pre-treated cells, pre-treatment with TPP-Niacin revealed diminished mitochondrial damage in the ARPE-19 cells (Figure 4C).

TPP-Niacin Increased Expression of OXPHOS and Mitochondrial Related Genes

To understand the molecular mechanisms of the protective effect of TPP-Niacin on mitochondrial biogenesis, the expression of mitochondrial respiration and mitochondrial dynamics genes were studied by real-time quantitative PCR (RT-qPCR). As expected, the expressions of OXPHOS component genes, ATP synthase subunit O (ATP5O), COX4I1(Cytochrome c oxidase subunit 4 isoform 1), Cytochrome c oxidase subunit 5B (COX5b), and NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5 (NDUFB5), were significantly increased by pre-treatment with TPP-Niacin in the ARPE 19 cells, as compared to H_2O_2 treatment alone and the control group (Figure 5A). As illustrated in Figure 5B, the mRNA expression levels of mitochondrial dynamics genes (fission 1 (FIS1), mitofusin 1 and 2 (MFN1 and 2)), mitochondrial DNA replication (polymerase (DNA directed), gamma (POLG)), and transcription gene (transcription factor a, mitochondrial (TFAM)), were significantly elevated by the TPP-Niacin compared to the H_2O_2 treated group. These results suggest that the upregulation of mitochondrial biogenesis genes act like in intermediating the protective effect of TPP-Niacin on H_2O_2-induced cell damage in ARPE-19 cells.

TPP-Niacin Increased Expression of Mitochondrial Biogenesis Related Genes via Upregulated PGC-1a/NRF2 Axis

The transcriptional coactivator, peroxisome-proliferator-activated receptor-gamma coactivator 1α (PGC-1α) is a potent moderator of mitochondrial function, including oxidative phosphorylation and mitochondrial biogenesis in RPE cells [24]. To further elucidate the involvement of PGC-1α in the protective effect of TPP-Niacin, the expression of PGC-1α related genes and antioxidant genes were assessed by RT-PCR. The expression of PGC-1α and PGC-1β was strongly upregulated after treatment with TPP-Niacin. Additionally, the gene expressions of ESRRA, FOXO1, FOXO3, NRF2, PPARα, and Sirt1 were significantly increased by TPP-Niacin (Figure 6A). Consistent with the results of ELISA (as shown in Figure 3), the TPP-Niacin (25 and 50 μM) pre-treatment increased the expressions of SOD1, SOD2, CAT, and GPX in ARPE-19 cells compared to the H_2O_2 treated cells. In addition, the NRF2 downstream target genes, NAD(P)H: quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), were also measured by RT-PCR. As shown in Figure 6B, the TPP-Niacin strongly upregulated the expression of HO-1 and NQO-1 but not that of NOX genes (data not shown) in the H_2O_2-treated ARPE-19 cells. These results suggest that the detoxified effects of TPP-Niacin may be due to its action as a ROS scavenger, by which it increases the expression level of the antioxidant enzyme, via reducing oxidative damage.

Discussion

Oxidative stress in the retina plays a major role in the pathogenesis of dry AMD. While antioxidant defence systems in the retinal cells are appropriate in normal states, strong oxidative stress disintegrates the normal antioxidant systems and result in irreparable damage to the retina. It has been reported that the use of additional antioxidants reduces oxidative stress and preserves retinal function while avoiding oxidative damage. [25, 26]. In addition, experimental and clinical studies suggest that consuming high doses of antioxidants, such as lutein, β-carotene, vitamins, and zinc supplements, are possible

protections for curtailing the progression of AMD and vision loss [2]. In the present study, we demonstrated the improved protective effects of TPP-Niacin for the first time; this is a mitochondrial targeting compound against oxidative damage in human RPE cells. In the mitochondrial level, the TPP-Niacin exerts improved protective effects via the mediation of the MMP and its related effector genes, including OXPHOS, mitochondrial dynamics, and mitochondrial DNA replication and transcription. Notably, TPP-Niacin is capable of supplying the prevention against oxidative damage by increment the expression level of antioxidant enzymes, mainly HO-1 and NQO-1, via the upregulation of PGC-1a and NRF2 in the ARPE-19 cells. Furthermore, TPP-Niacin provides better protection than niacin against oxidative damage in ARPE-19 cells, therefore underscoring the potential use of TPP-Niacin as a possible therapeutic agent for AMD, a disease initiated by cell death from oxidative stress and RPE dysfunction.

RPE cells are one of the types of cells that consume high amounts of energy, exist in the back of the photoreceptor cells, and have the most commonplace oxidative-damaged composition in the retina. H_2O_2 is a critical factor in producing oxidative damage and cell death in various cell types, including retinal cells [27]. In the present study, H_2O_2 was used to test ARPE-19 cells to generate oxidative stress and cell cytotoxicity to imitate the onset of dry AMD. As noted in the viability and LDH assays, pre-treatment with TPP-Niacin in the ARPE-19 cells significantly increased the cell viability against H_2O_2-induced cell death, whereas TPP-Niacin reduced the cell death from oxidative damage. Intriguingly, TPP-Niacin treatment alone was able to slightly increase the growth of ARPE-19 cells compared to the parent compound (Supplementary Figure 1A).

Intracellular accumulation of ROS is interconnected with oxidative stress and dysfunction of RPE cells [28]. Diminishments of intracellular ROS may shield the RPEs from oxidative damage [5, 29]. The results in this study confirmed that TPP-Niacin markedly diminished H_2O_2-induced intracellular ROS levels in RPE cells, as observed via DCFDA and DHE staining. There are major antioxidant enzymes, including Cu/Zn-superoxide dismutase (cytosolic SOD, SOD1), manganese superoxide dismutase (mitochondrial SOD, SOD2), catalase, and glutathione peroxidase (GPx). The SODs dismutase superoxide to oxygen and hydrogen peroxide, whereas catalase and GPx transform hydrogen peroxide into H_2O and O_2 [28, 29]. The present study demonstrated that pre-incubation with TPP-Niacin increased SOD1 and SOD2 compared to the H_2O_2 group, thus suggesting that TPP-Niacin may combat oxidative stress. Additionally, as a result of catalase and GPx, the TPP-Niacin significantly increased catalase and GPx activities in the decreased by H_2O_2 in ARPE-19 cells. These data indicate that TPP-Niacin may retain the ability to indirectly scavenge oxygen free radicals. Consequently, TPP-Niacin may reduce H_2O_2-induced oxidative stress in ARPE-19 cells by falling the intracellular ROS status and by eliminating oxygen free radicals. In addition, we observed that ARPE-19 cells pre-treated with niacin and TPP-Niacin markedly reduced H_2O_2-induced ROS production. As expected, the TPP-Niacin exerted a somewhat higher preventive effect against oxidative damage, as shown by a 10% increment in the cell viability and 17% decrement in ROS level compared to niacin-treated cells, respectively. The antioxidant activities of TPP-Niacin are at a slightly higher level compared to the parent compound, suggesting that TPP-Niacin is an effective derivative of the parent compound.

The pathological changes of mitochondrial-related dysfunction, including accumulation of ROS and superoxide in mitochondria and MMP (△Ψm) reduction, were discovered in AMD. [25]. In other mitochondrial targeting compounds [10, 14, 31], we observed that pre-treatment with TPP-Niacin significantly enhanced the MMP and improved the mitochondrial ultrastructure in a phenotypic analysis by EM, compared to H_2O_2 alone. Based on these data, we next analyzed gene expressions of mitochondria-related genes, such as OXPHOS subunits, mitochondrial dynamics, and mitochondrial DNA replication and transcription genes. Our results showed that TPP-Niacin significantly upregulated COX4I1, COX5B, NDUFB and MFN1, MFN2, TFAM, and POLG genes; thus, these mitochondrial specific effects of TPP-Niacin could lead to improved mitochondrial function and biogenesis against oxidative stress by H_2O_2.

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and -beta (PGC-1β) are transcriptional coactivators that control mitochondrial metabolism and function in various tissues [32], including the retina [27, 33, 34]. To intermediate their functions, the PGC-1α isoforms cooperate with transcription factors, such as estrogen-related receptor alpha (ESRRA), peroxisome proliferator-activated receptor α, γ (PPARα, γ), forkhead foxO1 and 3 (FOXO1), and nuclear respiratory factors 1 (NRF1) and Nfe212 (nuclear factor erythroid 2-related factor 2, NRF2) to control respiration, mitochondrial biogenesis, and expression of antioxidants [27, 35]. PGC-1α is necessitated for the generation of ROS scavenging enzymes, including SOD1, SOD2, GPx, and CAT [36, 37]. Recently, several studies have shown that superoxide dismutase 2 (SOD2), an enzyme detoxifying an excessive accumulation of mitochondrial ROS, was turned on by PGC-1α in RPE cells [27, 38]. Therefore, to determine the possible pathway of the protective effect of TPP-Niacin, we examined the gene expressions of PGC-1α related genes. We observed that PGC-1α and PGC-1β were robustly upregulated by TPP-Niacin compared to the H_2O_2-induced oxidative damage group. In addition, when examining potential downstream transcription factors responsible for these changes, ESRRA, FOXO1 and 3, and NRF1 and 2 were found to be upregulated by TPP-Niacin treatment.

On further investigating the possible mechanism associated with the protective ability of TPP-Niacin, it has appeared that the HO-1 and NQO-1 of downstream target genes of NRF2 signalling play major roles in the prevention of the cells from oxidative damage [39, 40]. Recently, many studies have reported that the activation of NRF2/HO-1 signalling is required for the alleviation of oxidative damage to RPE cells [40–45]. In this study, it was speculated that the anti-oxidative effects of TPP-Niacin might be combined with PGC-1α and NRF2 signalling. The results of the present study reveal that TPP-Niacin protects the ARPE-19 cells from H2O2-induced oxidative damage by activating the NRF2 signalling through upregulation of the expression of NRF2, NQO-1, and HO-1.

Initially, we thought that TPP-Niacin had an effect on nano-concentration state, as well as other mitochondrial targeting compounds, but TPP-Niacin showed antioxidant effects in the range of 10–200 μM. However, as shown in the comparison data with niacin, TPP-Niacin was more effective than its original chemical against oxidative damage in RPE cells. These results underscore TPP-Niacin as a more potent antioxidant against oxidative stress compared to niacin and suggest that its improved protective

effects are exerted via regulation of mitochondrial dynamics and antioxidant mechanisms. As many reported other research, it is well established that niacin exerts significant antioxidant, anti-inflammatory and anti-apoptotic activities in a variety of cells and tissues [19, 20, 48–50]. Our study so far has only been applied to focus on the improved antioxidant effect of TPP-Niacin, in terms of mitochondrial and ROS regulation. Further data collection would be needed to determine exactly how TPP-Niacin affects with antioxidant effect via mitochondrial biogenesis and dynamics.

In conclusion, this study shows that TPP-niacin is an improved protective antioxidant than niacin against oxidative damage to ARPE-19 cells via the reduction of ROS levels and protection against oxidative stress-induced cell death. The signal mechanisms by which TPP-Niacin presented such effects involve regulation of the mitochondrial quality control and transcriptional factors such as PGC-1α and NRF2, as well as a boost of antioxidant molecules. These results supply the first experimental evidence for TPP-Niacin as a possible therapeutic agent in the prevention of AMD.

Abbreviations

MMP: mitochondrial membrane potential; NAC: N-acetyl-cysteine; CCCP: carbonyl cyanide m-chlorophenyl hydrazone; H_2DCF-DA:2,7-dichlorofluorescein diacetate; DHE: dihydroethidium; ATP5O: ATP synthase subunit O ; COX4I1:Cytochrome c oxidase subunit 4 isoform 1; COX5b: Cytochrome c oxidase subunit 5B; NDUFB5: NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5; POLG: mitochondrial DNA replication (polymerase (DNA directed), gamma; TFAM: transcription factor a, mitochondrial; ESRRA: estrogen-related receptor alpha; PPARα, γ: peroxisome proliferator-activated receptor α, γ ; FOXO1: forkhead foxO1; and NRF1: nuclear respiratory factors 1; NRF2: Nfe212 (nuclear factor erythroid 2-related factor 2.

Declarations

Acknowledgments

Not applicable.

Authors' Contributions

M.H.K. and S.G. Y. conceptualization of the study; M.H.K. and D.H. K. performed the experiments; M.H.K. analyzed the data; M.H.K. wrote the paper; D.Y.K. and S.G.Y. reviewed and edited the manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Primer sequences used in this study

Target gene	Forward sequence (5’-3’)	Reverse sequence (5’-3’)
ATP5O	CGCTATGCCACAGCTCTTTA	AAGGCAGAAACGACTCCTTG
COX4I1	GGCATTGAAGGAGAAGGAGA	TCATGTCCAGCATCCTCTTG
COX5B	GAGGTGGTGTTCCCACTGAT	CAGACGACGCTGGTATTGTC
NDUFB5	CTTCCTCACTCGTGGCTTTC	TCTGGGACATAGCCTTCTGG
FIS1	GACATCCGTAAAGGCATCGT	ACAGCAAGTCCGATGAGTCC
MFN1	TGCCCTCTTGAGAGATGACC	TCTTTCCATGTGCTGTCTGC
MFN2	ATGCATCCCCACTTAAGCAC	GCAGAACTTTGTCCCAGAGC
TFAM	TAAGACTGCAAGCAGCGAAG	TTCTCAGTTTCCCAGGTGCT
POLG	TGCAGTGAGGAGGAGGAGTT	CCCAGGTAAGTGCCATGAGT
SOD1	GAAGGTGTGGGGAAGCATTA	CTTTGCCCAAGTCATCTGCT
SOD2	AAACCTCAGCCCTAACGGTG	GCCTGTTGTTCCTTGCAGTG
CAT	GATAGCCTTCGACCCAAGCA	AGAAGGCTGTTGTTCCGGAG
GPX1	AGTCGGTGTATGCCTTCTCG	CAAACTGGTTGCACGGGAAG
TXN2	TGGTGGCCTGACTGTAACAC	CACCGCTGACACCTCATACT
PRDX3	TGCATGTGGAAGAACGAGCT	TCCACTGAGACTGCGACAAC
PRDX5	AGGGTGTGCTGTTTGGAGTT	TCCACATTCAGGGCCTTCAC
PRDX6	CAGCTCGTGTGGTGTTTGTT	AGATGGGAGCTCTTTGGTGA
HMOX1	AGTCTTCGCCCCTGTCTACT	GCTTGAACTTGGTGGCACTG
NQO1	AAAGGACCCTTCCGGAGTAA	CGTTTCTTCCATCCTTCCAG
PGC1a	CAAGCAAAGGGAGAGGCAGA	ACCTGCGCAAAGTGTATCCA
PGC1b	TGGGCTGAGTTCTCCATTCT	TGAAGCTGCGATCCTTACCT
ESRRA	TCGCTGTCTGACCAGATGTC	AGGGCCAAGGCCTTTAGTAG
FOXO1	GCATCCATGGACAACAACAG	AGATGGCGGGTACACCATAG
FOXO3	CATCATGGCAAGCACAGAGT	GAGCGTGATGTTATCCAGCA
NRF1	CTTACAAGGTGGGGGACAGA	CAATGTCACCACCTCCACAG
NRF2	GCGACGGAAAGAGTATGAGC	ACGTAGCCGAAGAAACCTCA
PPARA	CCCTTTTTGTGGCTGCTATC	ATCCGACTCCGTCTTCTTGA
SIRT1	CCATGGCGCTGAGGTATATT	TCTCCATCAGTCCCAAATCC

GAPDH	ACCCAGAAGACTGTGGATGG	TTCTAGACGGCAGGTCAGGT

Figures

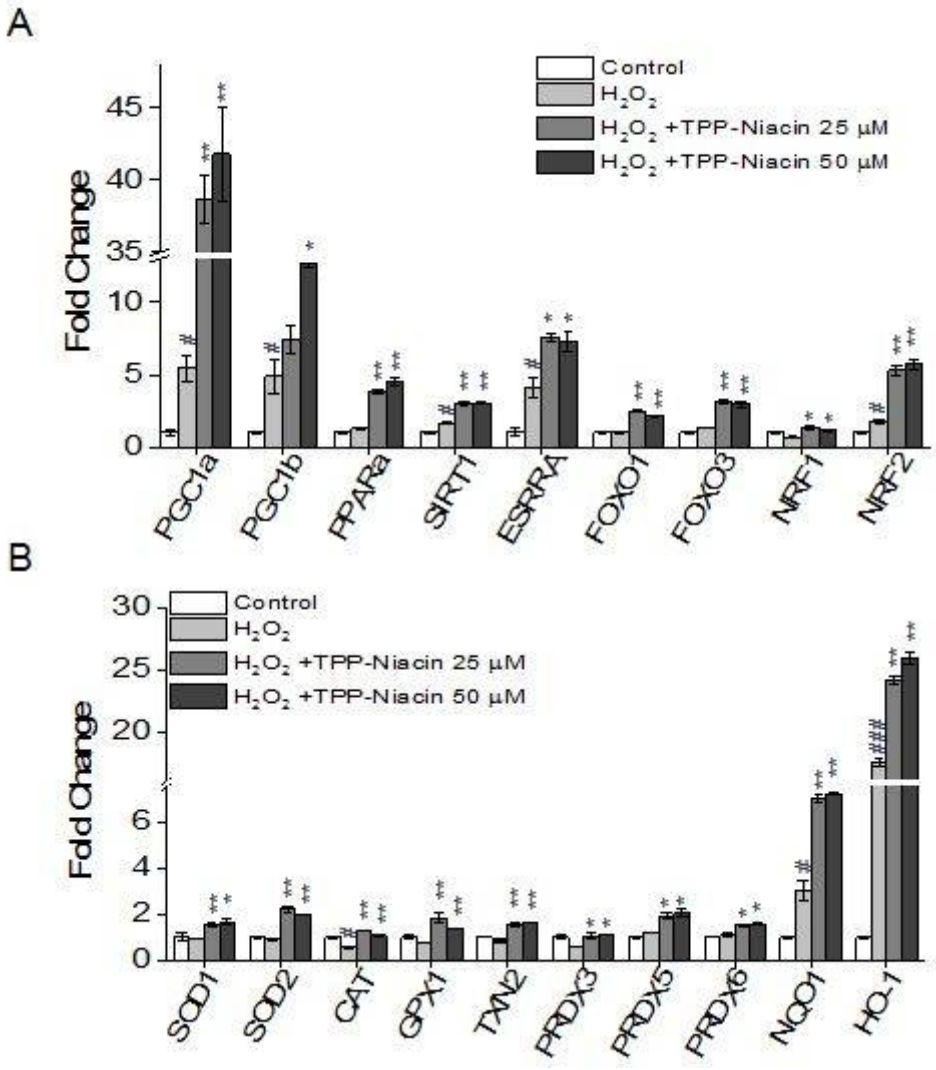
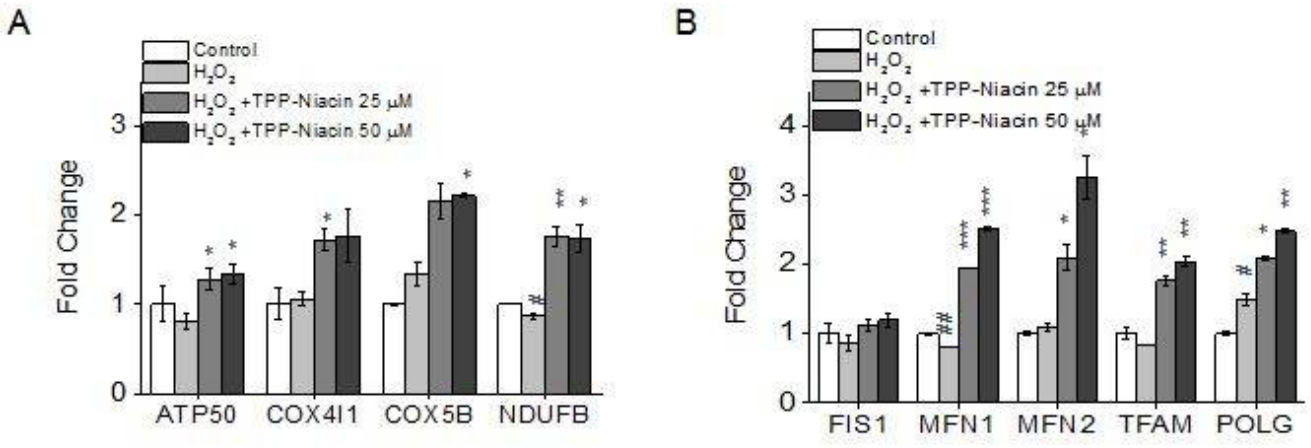


Figure 1

The involvement of PGC-1α/NRF2/NQO1/HO-1 axis for antioxidant effects of TPP-Niacin. Gene expression analysis by qPCR of transcription factors (a) and major antioxidant related genes (b). All gene expression data was analyzed using Student's t-test. Statistical significance is represented as follows: # P < 0.05, ### P < 0.01, versus control group; ∗P <0.05, ∗∗P <0.01, and ∗∗∗P <0.001, versus the H2O2-treated group.



A: OXPHOS components
B: mitochondrial dynamics and mitochondrial DNA replication & transcription

Figure 2

TPP-Niacin upregulated expression of OXPHOS target genes and mitochondrial dynamics genes in ARPE-19 cells. Relative gene expression of OXPHOS genes (a) and mitochondrial dynamic gene (FIS1, MFN1 and MFN2) and replication genes (TFAM and POLG) (b) in ARPE-19 treated for 2h with 25 or 50 μM TPP-Niacin and then incubated with or without 300 μM H2O2 for a further 24 h. # P < 0.05, ## P < 0.01, versus control group; *P < 0.05, **P < 0.01, ***P < 0.001 versus the H2O2-treated group were considered statistically significantly different.

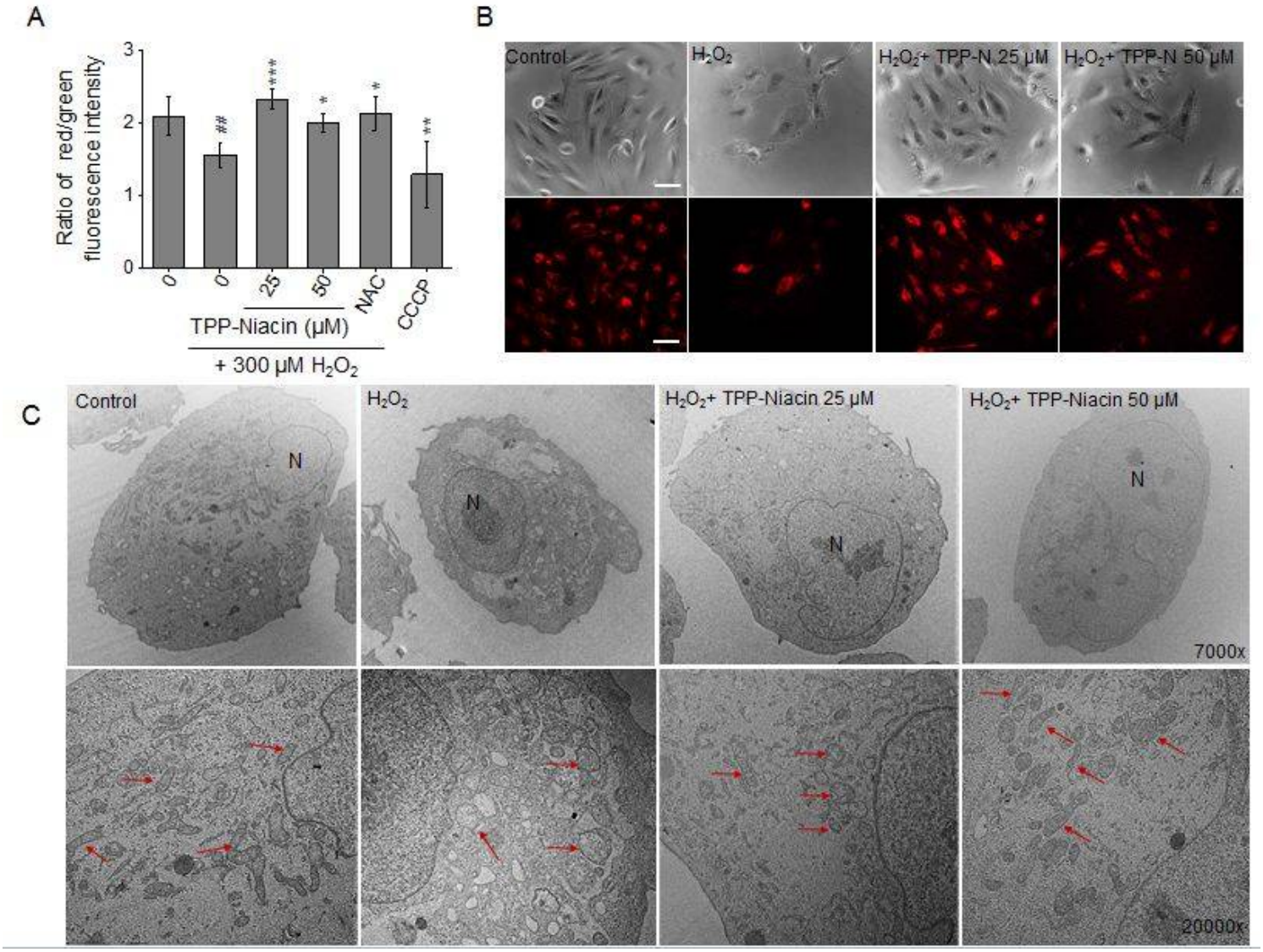


Figure 3

TPP-Niacin attenuated H2O2-induced mitochondrial membrane potential (△ψm) loss and mitochondrial morphology in ARPE-19 cells. After pre-treatment with 25 and 50 μM TPP-Niacin for 2 h, ARPE-19 cells were incubated with or without 300 μM H2O2 for another 24 h. Quantification of red/green fluorescence intensity was determined by JC-10 assay (a) and △ψm was determined by TMRE staining. (b) CCCP (40 μM) and NAC (4 mM) were used as positive and antioxidant control, respectively. (c) Representative electron microscope images of mitochondrial shape in the ARPE-19 cells treated without H2O2 or alone with 300 μM H2O2, or with 300 μM H2O2 and 25 or 50 μM TPP-Niacin for 24 hours. ## $P < 0.01$, versus control group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the H2O2-treated group were considered statistically significantly different. Scale bar represents 50 μm. TMRE: Tetramethylrhodamine, ethyl ester, CCCP: Carbonyl cyanide m-chlorophenyl hydrazine, NAC: N-acetyl-cysteine.

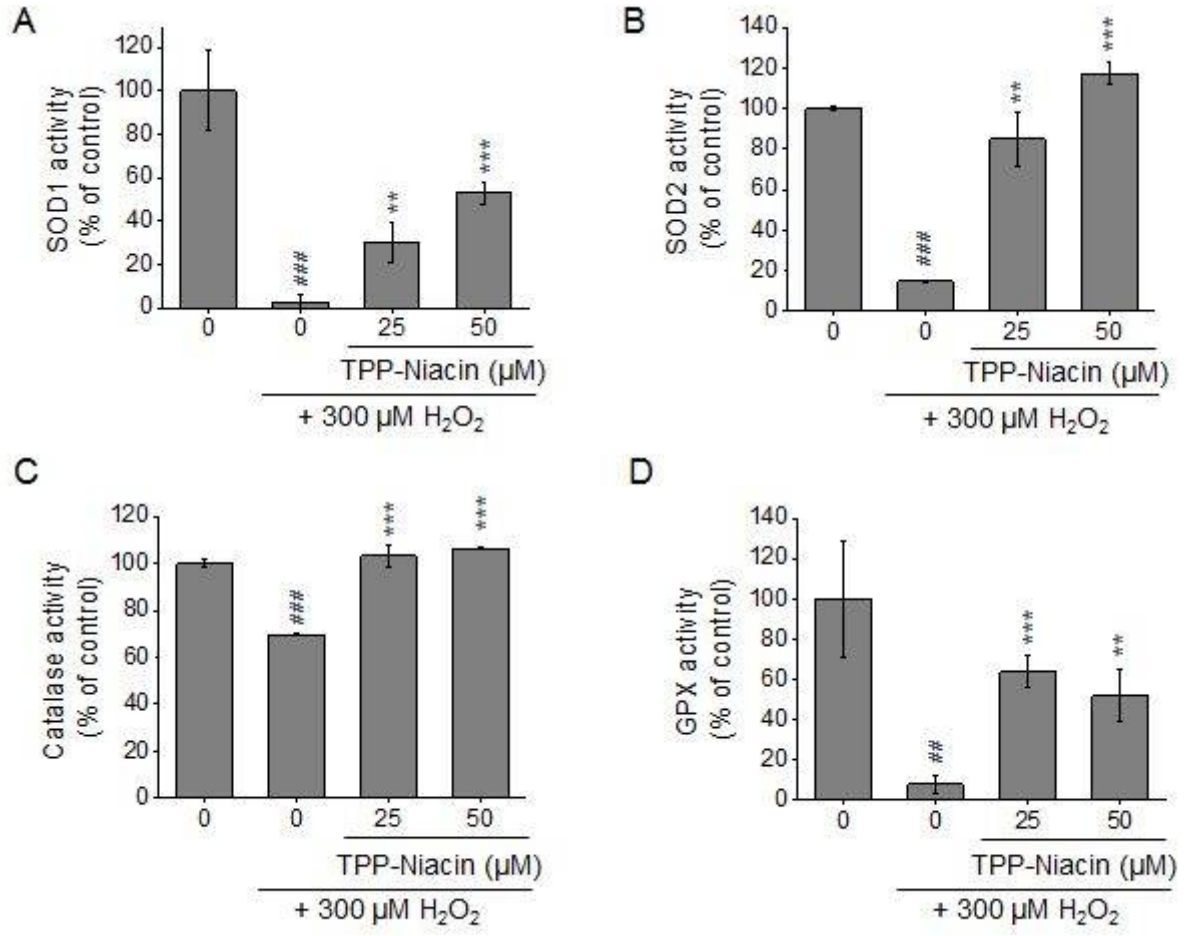
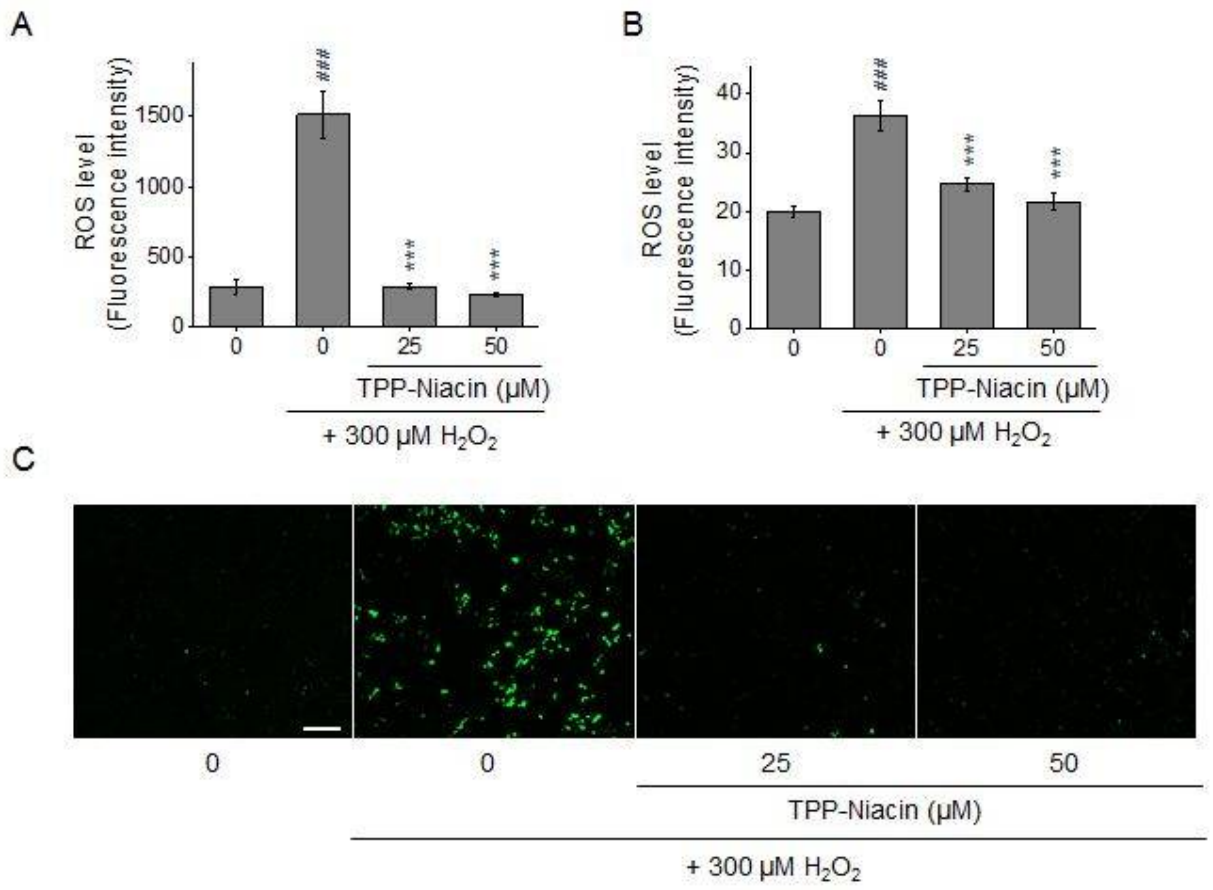


Figure 4

TPP-Niacin improved H2O2-induced decreasing antioxidant status in ARPE-19 cells. ARPE-19 cells were pretreated with TPP-Niacin at 25 and 50 μM for 2 h and then treated with H2O2 (300 μM) for 24 h. The levels of SOD1 (a), SOD2 (b) Catalase (c) and GPx activity (d) were measured to assess the level of antioxidant activity. ## $P < 0.01$, ### $P < 0.001$, versus control group; **$P < 0.01$, ***$P < 0.001$ versus the H2O2-treated group were considered statistically significant differences. SOD1 and 2: superoxide dismutase 1 and 2, and GPx: Glutathione peroxidase.



A: DCFDA, B: DHE

Figure 5

Protective effects of TPP-Niacin against H2O2-induced ROS generation in ARPE-19 cells. Cells were pre-treated with TPP-niacin at 25 and 50 μM or 0.1% DMSO (vehicle control) for 2 h and then incubated with or without 300 μM H2O2 for a further 24 h, and ROS generation was measured by the DCFDA (a) and DHE (b). Representative cell images were assessed by DCFDA staining. ### P < 0.001, versus control group; ***P < 0.001 versus the H2O2-treated group were considered statistically significant differences. The scale bar in (c) represents 100 μm. DCF-DA: dichlorofluorescein diacetate, DHE: dihydroethidium.

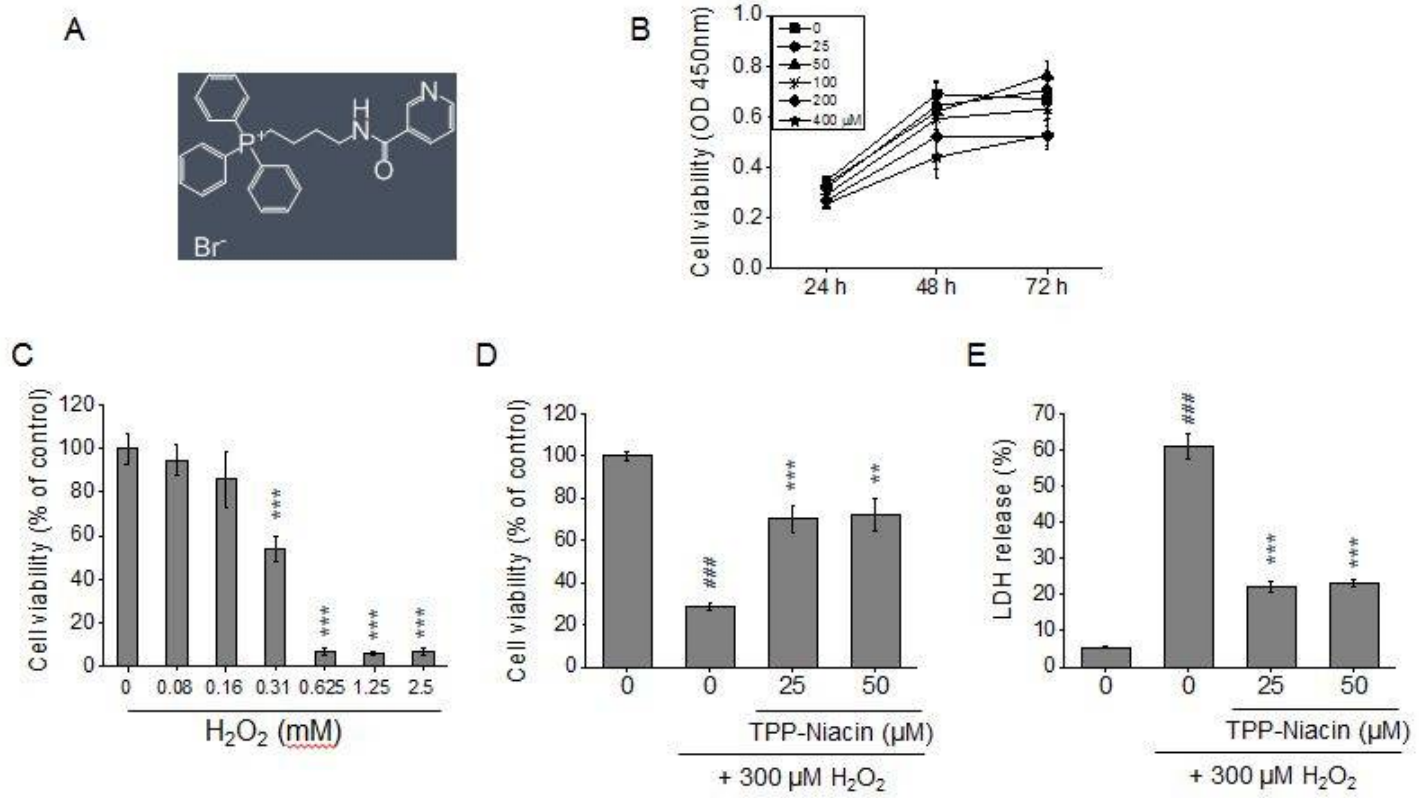


Figure 6

Protective effects of TPP-Niacin against H2O2-induced cytotoxicity in ARPE-19 cells. (a) chemical structure of TPP-Niacin. (b) Cells were treated with TPP-Niacin (25–400 μM) or 0.1% DMSO (vehicle control) for 24-72 h and cell viability was measured using the CCK-8 assay. (c) Cells were treated with H2O2 (0.08–2.5 mM) for 24 h and cell viability was measured. Cells were pre-treated with TPP-Niacin at indicated concentration or 0.1% DMSO (vehicle control) for 2 h and then incubated with or without 300 μM H2O2 for a further 24 h. Cell viability and the release of LDH were measured by CCK-8 assay (d) and LDH assay (e), respectively. ### P < 0.001, versus control group; **P < 0.01, ***P < 0.001 versus the H2O2-treated group were considered statistically significant differences.