

Research Article

Blockade of Cyclophilin D Attenuates Oxidative Stress-Induced Cell Death in Human Dental Pulp Cells

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Received 21 October 2018; Revised 3 January 2019; Accepted 16 February 2019; Published 4 April 2019

Academic Editor: Manuela Corte-Real

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Pathological stimuli, such as bacterial activity, dental bleaching, and nonpolymerized resin monomers, can cause death of dental pulp cells (DPCs) through oxidative stress- (OS-) induced mitochondrial dysfunction. However, the crucial molecular mechanisms that mediate such a phenomenon remain largely unknown. OS is characterized by the overproduction of reactive oxygen species (ROS), e.g., H_2O_2 , O_2^- , and $\cdot OH$. Mitochondria are a major source of ROS and the principal attack target of ROS. Cyclophilin D (CypD), as the only crucial protein for mitochondrial permeability transition pore (mPTP) induction, facilitates the opening of mPTP and causes mitochondrial dysfunction, leading to cell death. In the present study, we hypothesized that CypD-mediated mitochondrial molecular pathways were closely involved in the process of OS-induced death of human DPCs (HDPCs). We tested the phenotypic and molecular changes of HDPCs in a well-established OS model— H_2O_2 treatment. We showed that H_2O_2 dramatically reduced the viability and increased the death of HDPCs in a time- and dose-dependent manner by performing MTT, flow cytometry, and TUNEL assays and quantifying the expression changes of Bax and Bcl-2 proteins. H_2O_2 also induced mitochondrial dysfunction, as reflected by the increased mitochondrial ROS, reduced ATP production, and activation of mPTP (decreased mitochondrial membrane potential and enhanced intracellular Ca^{2+} level). An antioxidant (N-acetyl-L-cysteine) effectively preserved mitochondrial function and significantly attenuated H_2O_2 -induced cytotoxicity and death. Moreover, H_2O_2 treatment markedly upregulated the CypD protein level in HDPCs. Notably, genetic or pharmacological blockade of CypD significantly attenuated H_2O_2 -induced mitochondrial dysfunction and cell death. These findings provided novel insights into the role of a CypD-dependent mitochondrial pathway in the H_2O_2 -induced death in HDPCs, indicating that CypD may be a potential therapeutic target to prevent OS-mediated injury in dental pulp.

1. Introduction

Dental pulp is highly vulnerable to various physicochemical and microbiological stimuli, such as acute injury, bacterial activity and metabolites, dental bleaching, and nonpolymerized resin monomers [1–3]. These stimuli can cause dental

pulp cell (DPC) death, eventually leading to irreversible pulp inflammation and necrosis [1]. One main pathogenic mechanism of these stimuli is oxidative stress- (OS-) mediated damage in DPCs [4, 5]. OS is characterized by the excessive generation of reactive oxygen species (ROS), e.g., H_2O_2 , O_2^- , and $\cdot OH$ [6]. OS stimulated by dental

composites and lipopolysaccharides has been reported to induce cell cycle alteration and death of human DPCs (HDPCs) [7]. During dental bleaching, exogenous hydrogen peroxide (H_2O_2) released from the bleaching solution can result in the cell death of DPCs [5].

Mitochondria are the main resource and also the major attacking target of ROS [8]. In response to the abovementioned pathological stimuli, the excessively generated ROS will cause rapid depletion of antioxidants and then induce oxidative damage to mitochondria, which subsequently lead to mitochondrial dysfunction in HDPCs [9]. Such a cell death can be attenuated by the application of mitochondria-specific antioxidants [10]. These results indicate that mitochondrial dysfunction is highly involved in OS-induced HDPC death. However, the underlying molecular mechanisms remain largely unknown.

The mitochondrial permeability transition pore (mPTP), assembled between the inner and outer mitochondrial membranes, opens with the relatively severe disturbance of intracellular redox and/or Ca^{2+} homeostasis [11, 12]. The mPTP opening can lead to a solute exchange between mitochondrial matrix contents and the surrounding cytoplasm, which is commonly linked to mitochondrial dysfunction [13]. Cyclophilin D (CypD) is a critical protein for mPTP opening [14]. CypD-dependent mPTP opening has been shown to play a key role in ROS-induced mitochondrial dysfunction [15] and cell death [14]. The pharmacological inhibition or genetic ablation of CypD can rescue mitochondrial dysfunction and cell damage induced by OS [16]. However, it remains unknown whether the CypD-dependent mitochondrial pathway is involved in the OS-mediated death of HDPCs. In this study, we aimed to identify the potential role of CypD in the regulation of mPTP and mitochondrial dysfunction in the OS-induced HDPC death.

2. Materials and Methods

2.1. Informed Consent and Ethical Approval. The human research commission of the School and Hospital of Stomatology, Wenzhou Medical University, Wenzhou, China (2018001), gave approval to the study. For the human tooth collection, a written informed consent was obtained from all the subjects.

2.2. Reagents. Cell culture medium and additional supplements were bought from Life Technologies (Grand Island, NY, USA). The antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The chamber slides and 4',6-diamidino-2-phenylindole (DAPI) were from Invitrogen (Carlsbad, CA, USA). MitoSOX Red, TMRM, and MitoTracker Green (MTGreen, Molecular Probes) were from Life Technologies (Grand Island, NY, USA). A terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling (TUNEL) kit was from Roche (Mannheim, BW, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), an annexin V-fluorescein isothiocyanate (FITC) detection kit, H_2O_2 , and N-acetylcysteine (NAC) were from Sigma-Aldrich (St. Louis, MO, USA). Cyclosporine A (CsA) was from Cell Signaling Technology (Beverly, MA,

USA). An adenosine triphosphate (ATP) assay kit was from Beyotime Institute of Biotechnology (Shanghai, China).

2.3. Cell Culture, Characterization, and Treatment. HDPCs were isolated from the dental pulp tissues of noncarious third molars and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco, Grand Island, NY, USA). Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. For HDPC lineage characterization, the morphological analysis was performed, and the cells were immunocytochemically stained for vimentin and keratin. The working concentrations of the compounds were as follows: NAC (2.5 mM) and CsA (2 μ M). The final concentration of dimethyl sulfoxide (DMSO) in the culture was less than 0.5% in all the experiments. The cells with or without H_2O_2 were treated with the indicated test compounds according to the experimental protocol.

2.4. Cell Viability. HDPCs were seeded in 96-well plates and treated with the indicated reagents. Then, HDPCs were incubated with 10 μ L MTT solution (5 mg/mL). After 4 h, the formazan crystals were dissolved by DMSO. The plates were then measured in a microplate reader. The optical density (OD) of the control group in MTT was taken as 100% viable.

2.5. siRNA-Mediated CypD Knockdown in HDPCs. CypD siRNA targeting human peptidylprolyl isomerase F (PPIF) and the negative control (NC) were purchased from RiboBio (Guangdong, China) and transfected in HDPCs with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.6. Measurement of Cell Death by Flow Cytometry and TUNEL Assays. The cell death was detected by annexin V labeled with FITC. Propidium iodide (PI) was used to determine cell necrosis. After exposure to various experimental conditions, the cells were trypsinized and labeled with fluorochromes, and then, cytofluorometric analysis was performed with a FACScan (Becton Dickinson, NY, USA). For the TUNEL assays, different groups of cells were grown on a coverslip, incubated with a TUNEL reaction mixture, counterstained with DAPI, and observed under a fluorescence microscope.

2.7. Western Blot Analyses. HDPCs were collected and lysed in the cell lysis buffer. Proteins were separated by electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane. Proteins bound by primary antibodies were visualized with an appropriate secondary antibody. The protein bands were detected using the Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA) and quantified using NIH ImageJ software.

2.8. Intercellular ATP Level Determination. The ATP levels were detected by an ATP detection kit according to the manufacturer's instructions. The data was measured via a luminescence plate reader.

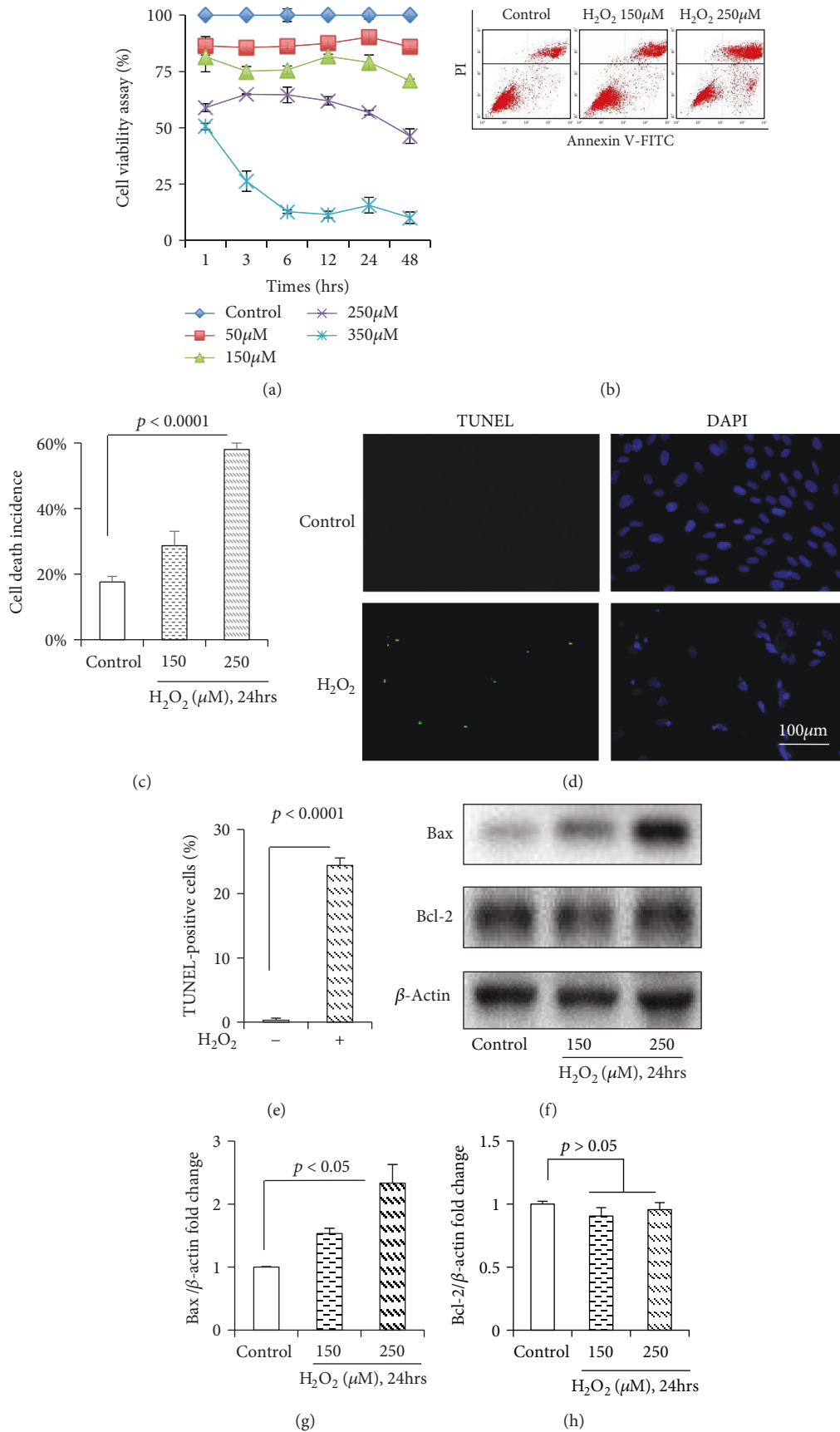


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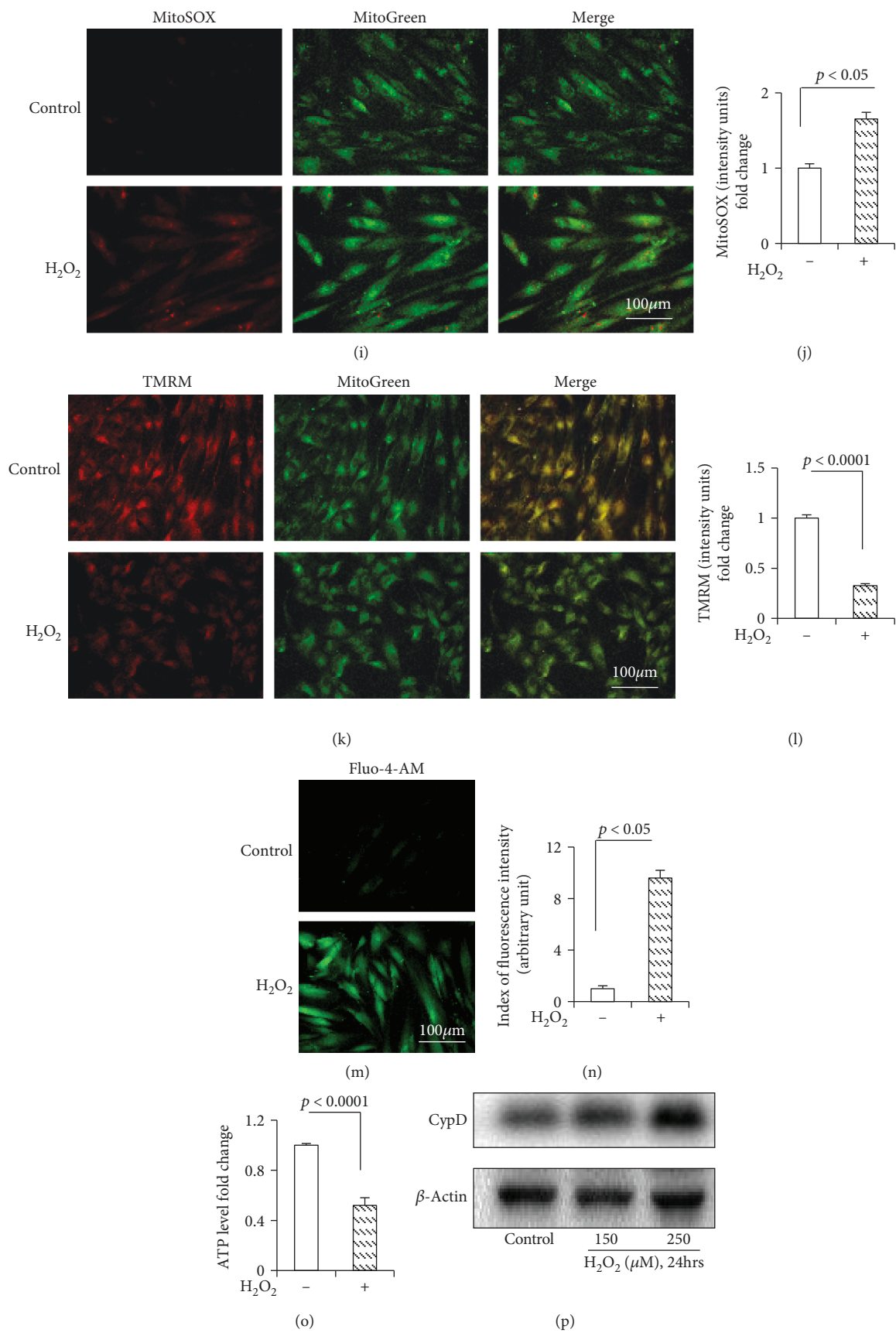


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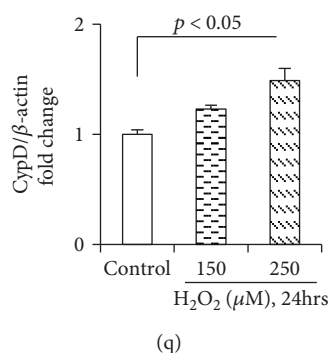


FIGURE 1: H₂O₂-induced cell death and mitochondrial dysfunction in the HDPCs. (a) Cell viability was determined by MTT reduction in the HDPCs with or without the presence of H₂O₂. (b, c) Flow cytometric quantification of cell death. HDPCs were treated for 24 h with culture medium or H₂O₂ (150 μM, 250 μM). (d, e) TUNEL staining and assay. (f) Representative immunoreactive bands with different densities for Bcl-2 and Bax in the HDPCs in the presence of H₂O₂. Quantification of immunoreactive bands for Bax (g) and Bcl-2 (h) relative to β-actin. Representative images showing MitoSOX staining (i) and quantification (j) in the indicated groups. Representative images with TMRM staining (k) and quantification (l) in the indicated groups. Representative images showing Fluo-4-AM staining (m) and quantification (n) in the indicated groups. ATP (o) in the indicated groups. (p) Densitometry of immunoreactive bands for CypD in the HDPCs in the presence of H₂O₂. (q) Quantification of immunoreactive bands for CypD relative to β-actin. HDPCs were treated for 24 h with H₂O₂ (250 μM) (+) or culture medium (-). Data represent the mean values ± SD of three independent experiments.

2.9. Functional Imaging Assays. After treatment with the indicated reagents, the cells were incubated with MitoSOX (2.5 μM) or TMRM (100 nM). Images were captured under the fluorescence microscope. The NIH ImageJ software was used to measure and quantify the fluorescence signals. More than 100 clearly identifiable mitochondria in 10-15 randomly selected cells per experiment were measured in three independent experiments [17, 18].

2.10. Detection of Ca²⁺ Level. The cells were treated with the indicated reagents. Then, the cells were incubated with 10 μM Fluo-4-AM (Beyotime, Shanghai, China), a Ca²⁺-sensitive fluorescent probe. The Ca²⁺ level was detected under a fluorescence microscope.

2.11. Data Analysis. Data were presented as the mean of three independent replicates ± standard deviation (SD) and considered significant at $p < 0.05$. Statistical analysis was performed using StatView software (version 5.0.1, SAS Institute, USA). For comparisons between the multiple groups, one-way ANOVA was used followed by individual post hoc Fisher tests when applicable.

3. Results

3.1. H₂O₂ Induced Cell Death and Mitochondrial Dysfunction in HDPCs. The cells around the pulp tissue, which showed a spindle shape and fibroblast-like morphology after being cultured for 5 days (Supplementary Figure 1a), were characterized as primary HDPCs. In addition, the cells exerted strong character of proliferation (Supplementary Figure 1b) and showed mesenchymal origin by the positive expression of vimentin in the cytoplasm but were negative for keratin (Supplementary Figures 1c and 1d).

As shown in Figure 1(a), H₂O₂ reduced HDPC viability in a time- and dose-dependent manner. The annexin/PI staining assay revealed a significant enhancement in the

incidence of cell death. 250 μM H₂O₂ markedly enhanced the rate of cell death compared with the control group (Figures 1(b) and 1(d)). The H₂O₂-induced DNA damage was further confirmed by the TUNEL staining (Figures 1(d) and 1(e)). Compared with the control group, H₂O₂ remarkably increased the protein level of proapoptotic Bax but did not influence antiapoptotic Bcl-2 (Figures 1(f) and 1(h)).

For the parameters of mitochondrial dysfunctions, H₂O₂ significantly increased the mitochondrial ROS (mtROS) level (MitoSOX staining) (Figures 1(i) and 1(j)) and decreased the mitochondrial membrane potential (MMP) (TMRM staining) (Figures 1(k) and 1(l)) in HDPCs. Furthermore, H₂O₂ markedly increased the intracellular Ca²⁺ level (Figures 1(m) and 1(n)) and reduced the ATP level (Figure 1(o)). In addition, H₂O₂ also significantly upregulated the expression of CypD (Figures 1(p) and 1(q)).

3.2. NAC Attenuated H₂O₂-Induced Cell Death and Mitochondrial Dysfunction in HDPCs. NAC, a nonspecific antioxidant, observably preserved the cell viability in HDPCs treated with H₂O₂ (Figure 2(a)) and significantly mitigated cell death (TUNEL staining) (Figures 2(b) and 2(c)) and Bax expression (Figures 2(d) and 2(e)). NAC attenuated the effects of H₂O₂ by significantly suppressing mtROS (Figures 2(f) and 2(g)), increasing MMP (Figures 2(h) and 2(i)), decreasing the intracellular Ca²⁺ level (Figures 2(j) and 2(k)), and enhancing the ATP level (Figure 2(l)). NAC also decreased the CypD expression (Figures 2(m) and 2(n)).

3.3. Inhibition of CypD by CsA Reversed H₂O₂-Induced Cell Death and Mitochondrial Dysfunction in HDPCs. CsA, a pharmaceutical inhibitor of CypD, significantly inhibited the H₂O₂-induced cytotoxicity and death, as was evident by the MTT assay (Figure 3(a)) and TUNEL staining (Figures 3(b) and 3(c)), respectively. Furthermore, CsA significantly downregulated the expression level of Bax and CypD (Figures 3(d) and 3(f)) and abrogated mtROS (Figures 3(g) and 3(h)) and

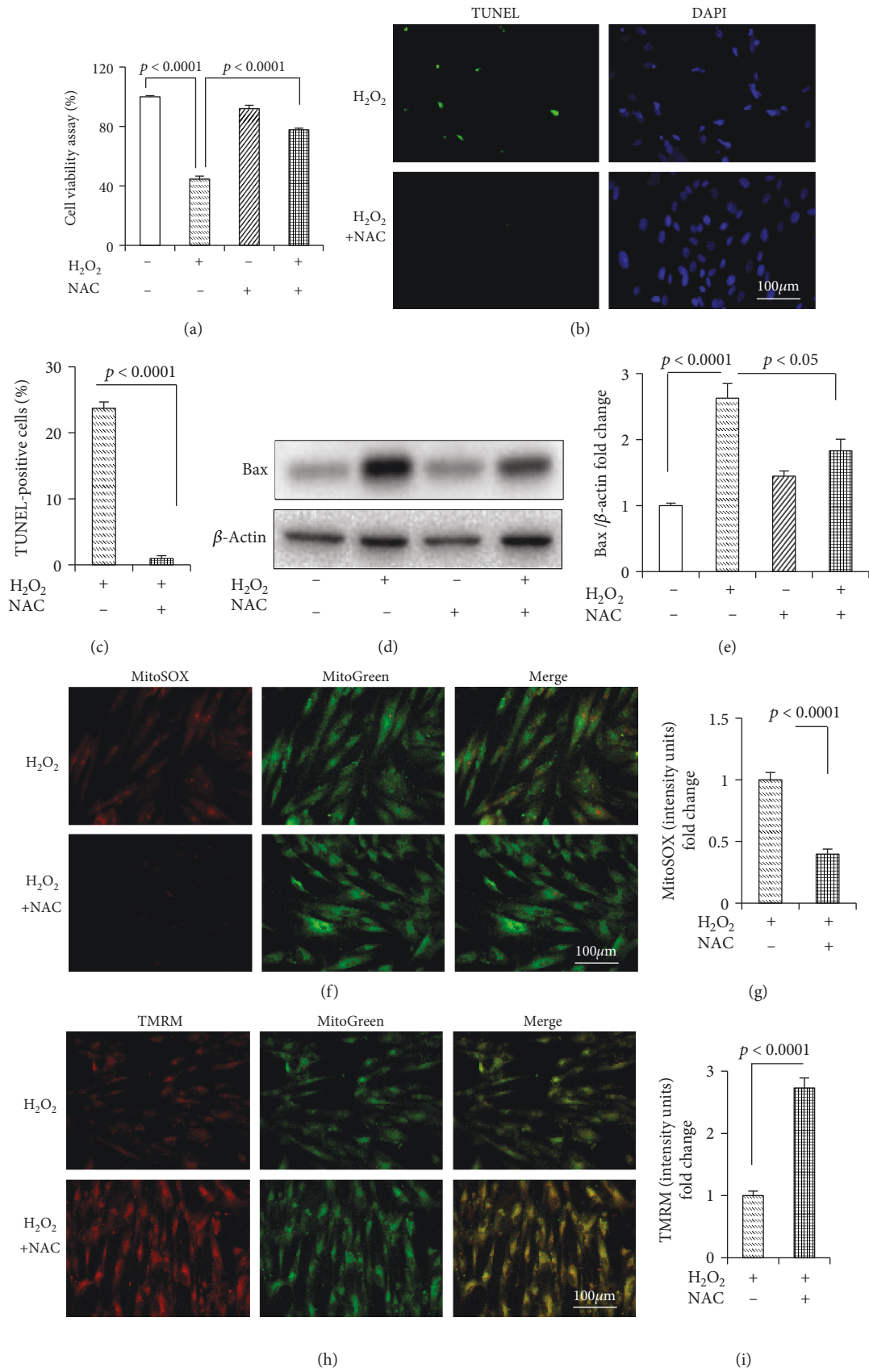


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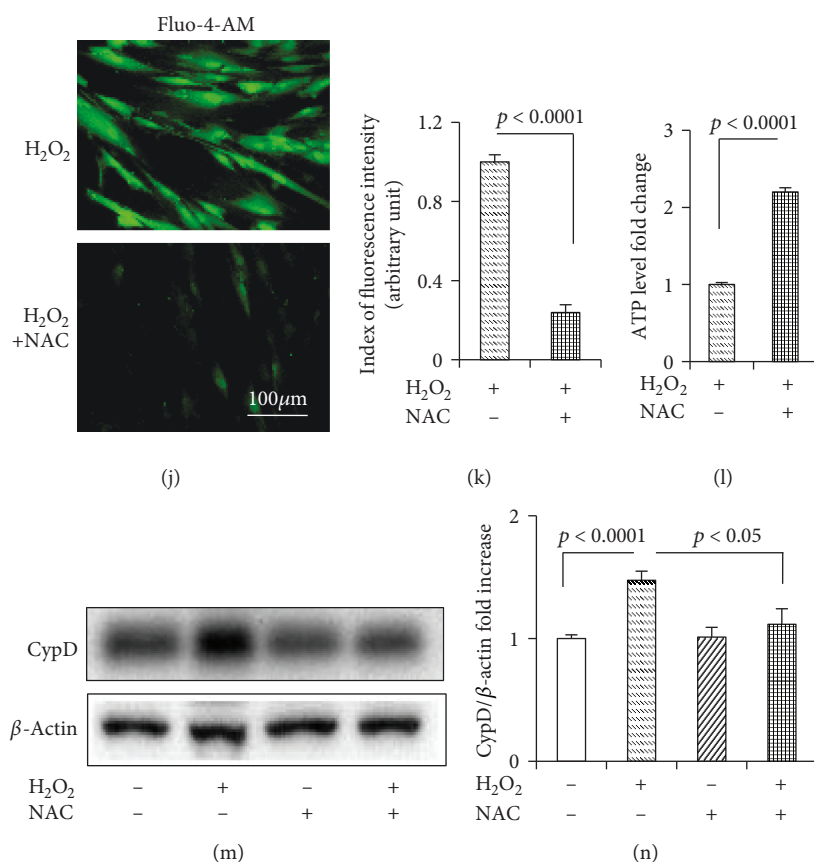


FIGURE 2: NAC attenuated H_2O_2 -induced cell death and mitochondrial dysfunction in the HDPCs. (a) Cell viability determined by MTT reduction in the HDPCs in the presence of H_2O_2 with or without NAC. (b) TUNEL staining and (c) assay after NAC treatment. (d) Representative immunoreactive bands for Bax in the HDPCs with (+) or without (-) NAC treatment in the presence of H_2O_2 (+) or culture medium (-). Quantification of immunoreactive bands for Bax (e) relative to β -actin. Representative images showing MitoSOX staining (f) and quantification (g) in the indicated groups. Representative images showing TMRM staining (h) and quantification (i) in the indicated groups. Representative images showing Fluo-4-AM staining (j) and quantification (k) in the indicated groups. (l) ATP level fold change in the indicated groups. (m) Densitometry immunoreactive bands for CypD in the HDPCs with (+) or without (-) NAC treatment in the presence of H_2O_2 (+) or culture medium (-). (n) Quantification of immunoreactive bands for CypD relative to β -actin. HDPCs were treated for 24 h with (+) or without (-) NAC (2.5 mM) in the presence of H_2O_2 (250 μ M) (+) or culture medium (-). Data represent the mean values \pm SD of three independent experiments.

the intracellular Ca^{2+} level (Figures 3(k) and 3(l)). CsA also effectively increased the MMP (Figures 3(i) and 3(j)) and ATP level (Figure 3(m)).

3.4. Blockade of CypD by siRNA Mitigated H_2O_2 -Induced Cell Death and Mitochondrial Dysfunction in HDPCs. The genetic knockdown of CypD with siRNA-PPIF was further conducted to validate the effect of CypD on HDPC death and mitochondrial dysfunction. Western blotting proved that siRNA-PPIF significantly downregulated the CypD expression (Figures 4(a) and 4(b)). We found that siRNA-PPIF significantly attenuated the H_2O_2 -induced cytotoxicity (Figure 4(c)) and death (Figures 4(d) and 4(e)). siRNA-PPIF significantly inhibited the H_2O_2 -induced expression of Bax and CypD (Figures 4(f) and 4(h)) and alleviated the mtROS and intracellular Ca^{2+} level (Figures 4(i), 4(j), 4(n), and 4(o)). siRNA-PPIF also protected the mitochondrial function from the adverse effect of H_2O_2 (Figures 4(k) and 4(m)).

4. Discussion

Substantial evidence has shown that many pathogenic stimuli can cause significant oxidative damage, leading to inflammation and necrosis in dental pulp tissues. However, hitherto, the underlying molecular mechanisms accounting for the OS-induced death of HDPCs remain elusive. In this study, we, for the first time, reported the key role of CypD in the OS-induced death of HDPCs. We found that pharmacological blockage and genetic reduction of CypD could significantly attenuate the oxidative damage to HDPCs. Our findings delineated novel insights into the crucial role of CypD-dependent mPTP opening and mitochondrial dysfunction in the OS-induced HDPC death.

Excessive ROS production can be induced in dental pulp by pathogenic stimuli, such as bacterial metabolite, dental bleaching, and unpolymerized resin monomers [2, 3, 19]. H_2O_2 is the most stable and commonly existing form of ROS and is widely used to establish cell OS injury

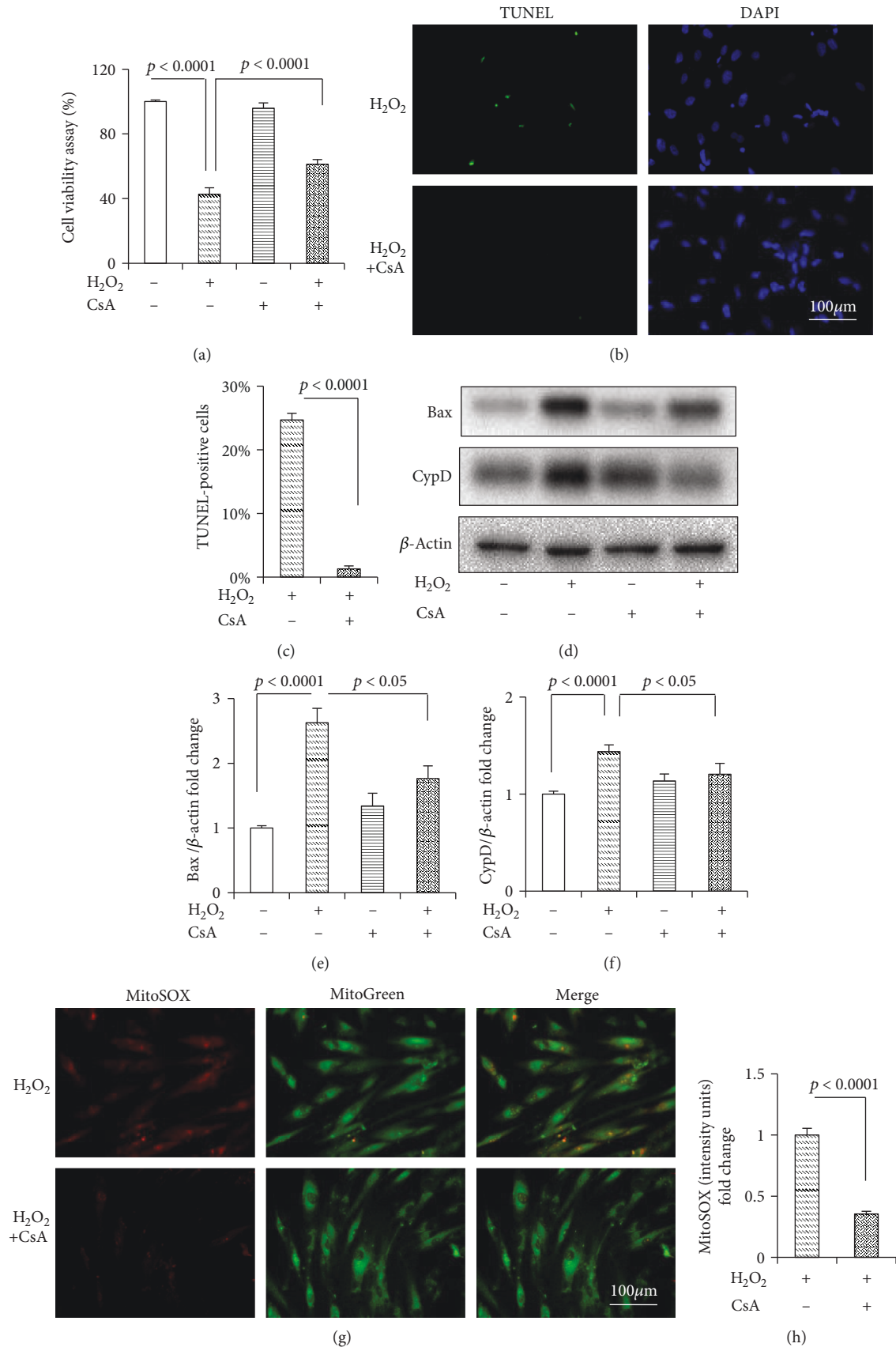


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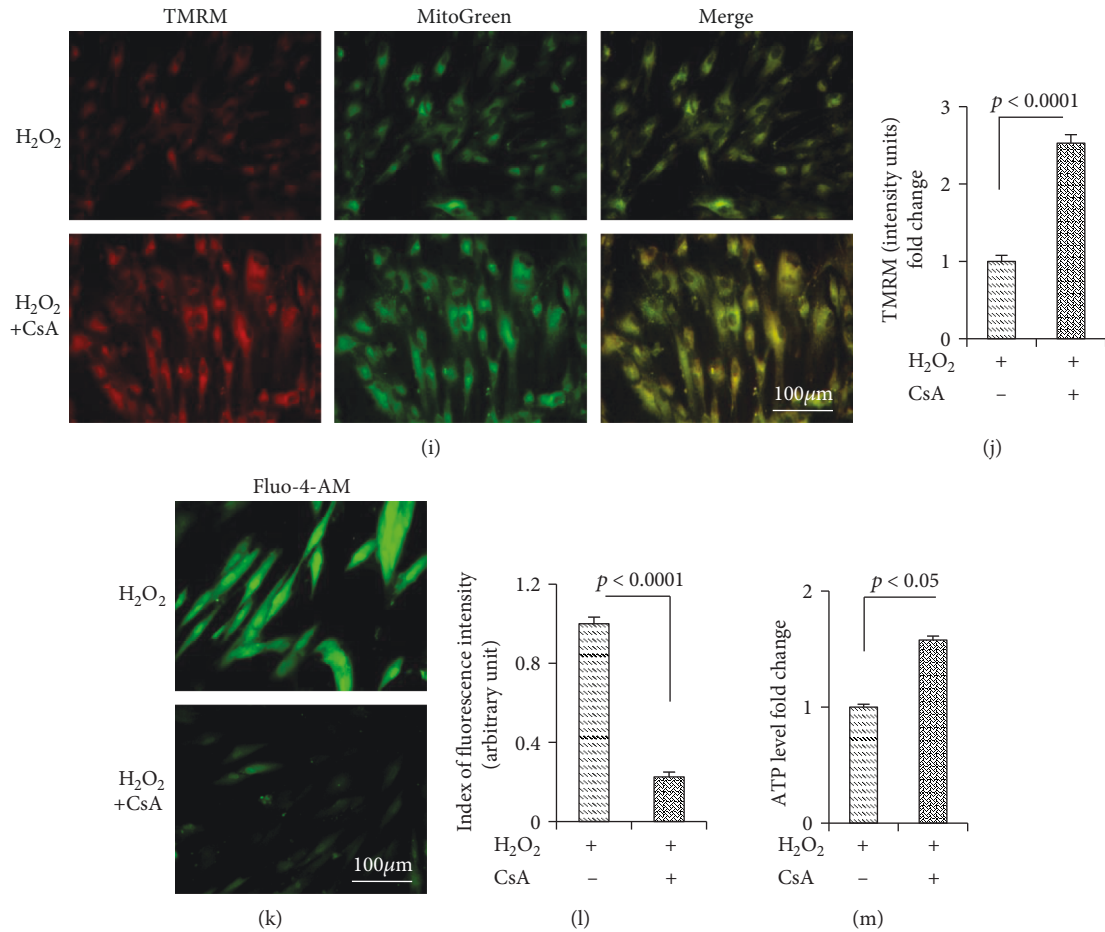


FIGURE 3: CsA attenuated H₂O₂-induced cell death and mitochondrial dysfunction in the HDPCs. (a) Cell viability determined by MTT reduction in the HDPCs in the presence of H₂O₂ with or without CsA. (b) TUNEL staining and (c) assay after CsA treatment. (d) Representative immunoreactive bands for Bax and CypD in the HDPCs with (+) or without (-) CsA treatment in the presence of H₂O₂ (+) or culture medium (-). Quantification of immunoreactive bands for Bax (e) and CypD (f) relative to β -actin. Representative images showing MitoSOX staining (g) and quantification (h) in the indicated groups. Representative images showing TMRM staining (i) and quantification (j) in the indicated groups. ATP (m) in the indicated groups. Representative images showing Fluo-4-AM staining (k) and quantification (l) in the indicated groups. HDPCs were treated for 24 h with (+) or without (-) CsA (2 μ M) in the presence of H₂O₂ (250 μ M) (+) or culture medium (-). Data represent the mean values \pm SD of three independent experiments.

models [20, 21]. In this study, we found that H₂O₂ significantly decreased the viability of HDPCs. Furthermore, H₂O₂ dramatically enhanced the death of HDPCs in comparison with the control group, as indicated by the results of flow cytometry and TUNEL assays.

Mitochondrial outer membrane permeation (MOMP) is a key event in apoptotic cell death, which is controlled by the Bcl-2 family proteins [22]. Bax and Bcl-2, Bcl-2 family proteins, are important in regulating apoptotic cell death. Bax is upregulated in response to DNA damage [23] and regulates mitochondria-dependent cell death [24]. In response to apoptotic stimuli, Bax oligomerizes and forms pores perforating the outer mitochondrial membrane [25, 26]. MOMP occurs during pore formation, and the size of the pore can vary according to the number of recruited Bax dimers [27]. Bcl-2 plays an important part in promoting cellular survival and bating the actions of proapoptotic proteins [28]. Bcl-2 antagonizes MOMP by blocking Bax

oligomerization and pore-forming activity [29, 30]. Our immunoblot analyses showed that H₂O₂ significantly increased the expression of proapoptotic protein Bax in HDPCs, which was consistent with the previous findings from osteoblastic cells [18] and human fetal lung fibroblast cells [31]. On the other hand, different from these studies, we found that Bcl-2 was not significantly affected by H₂O₂, which might be due to the different cell types.

Increased ROS triggers cell death by inducing mitochondrial dysfunction [17, 32, 33]. NAC primarily abrogates mtROS and therefore mitigates myocardial cell death [20]. In the current study, H₂O₂ resulted in significant mitochondrial dysfunction, which was indicated by the increased mtROS, enhanced intracellular Ca²⁺ level, and decreased ATP level and MMP in HDPCs. NAC, in this study, not only blunted OS but also attenuated mitochondrial dysfunction in HDPCs. These results further confirmed that ROS-induced mitochondrial dysfunction played a key role in

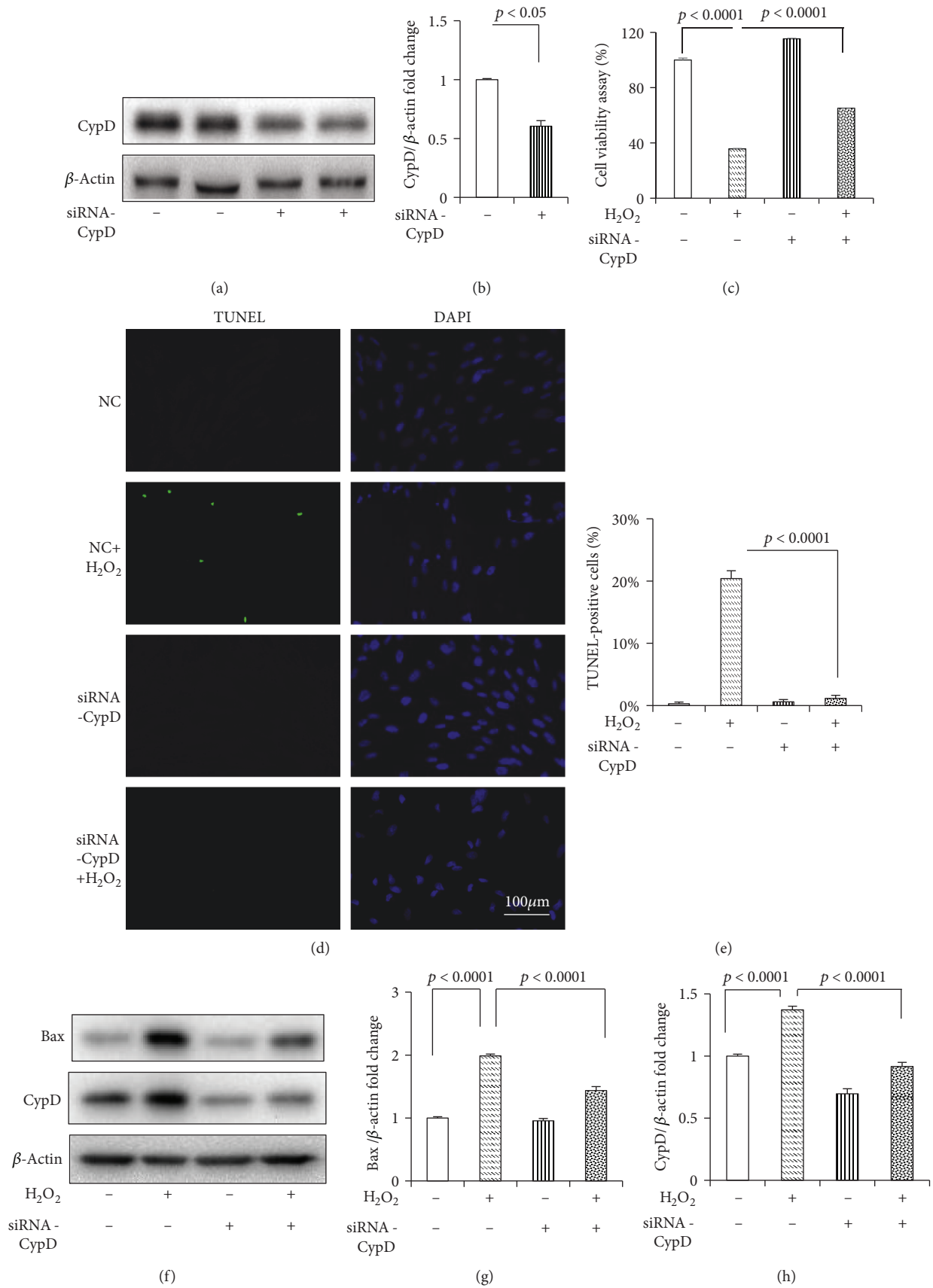
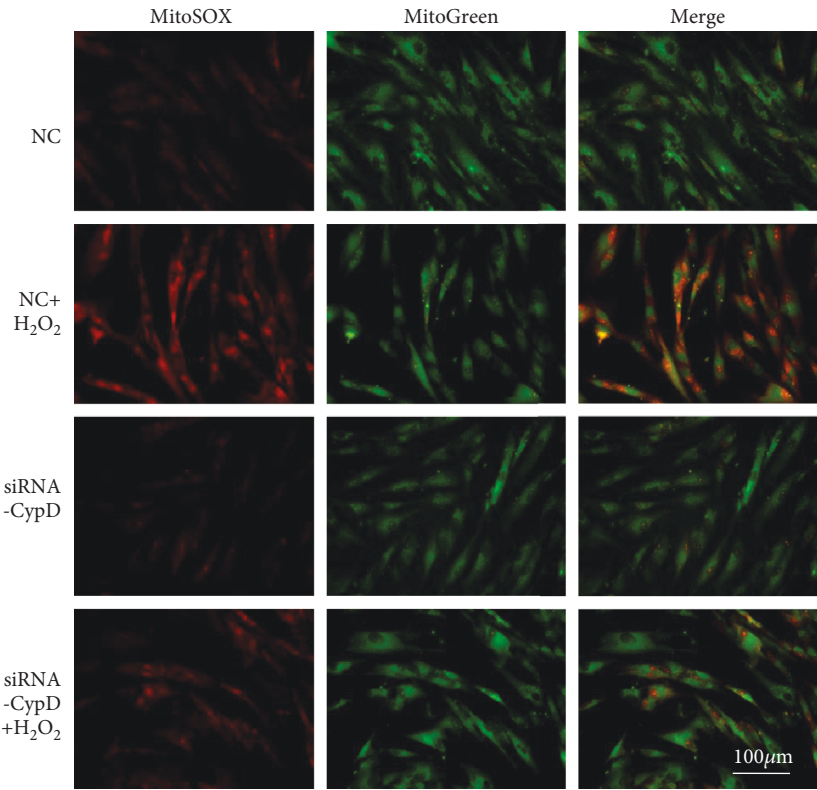
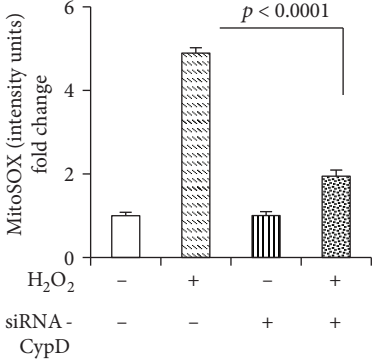


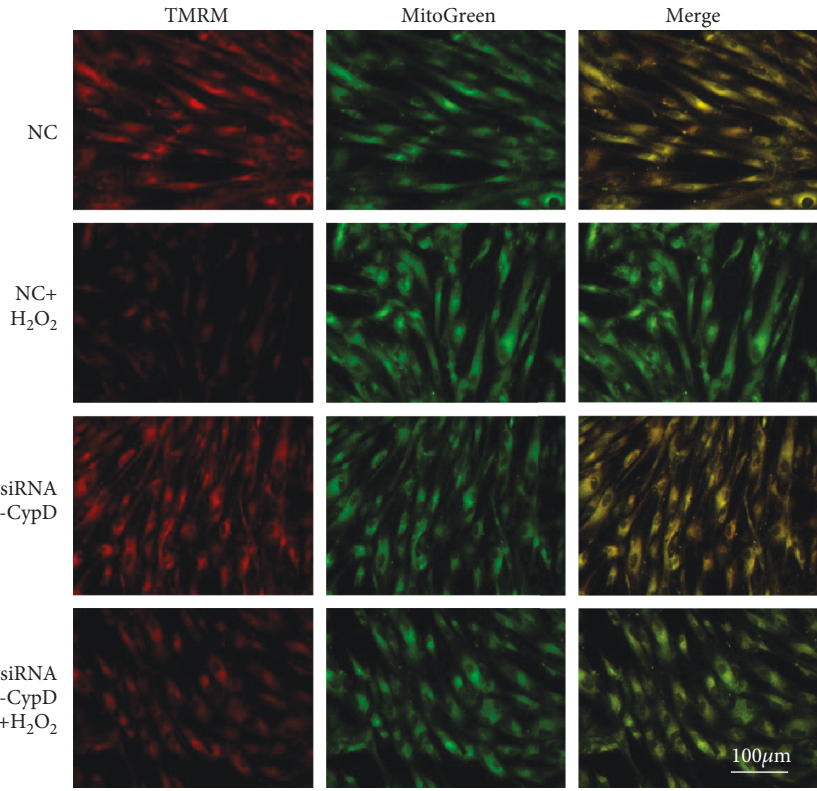
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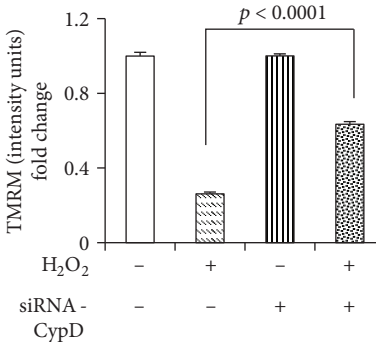
(i)



(j)



(k)



(l)

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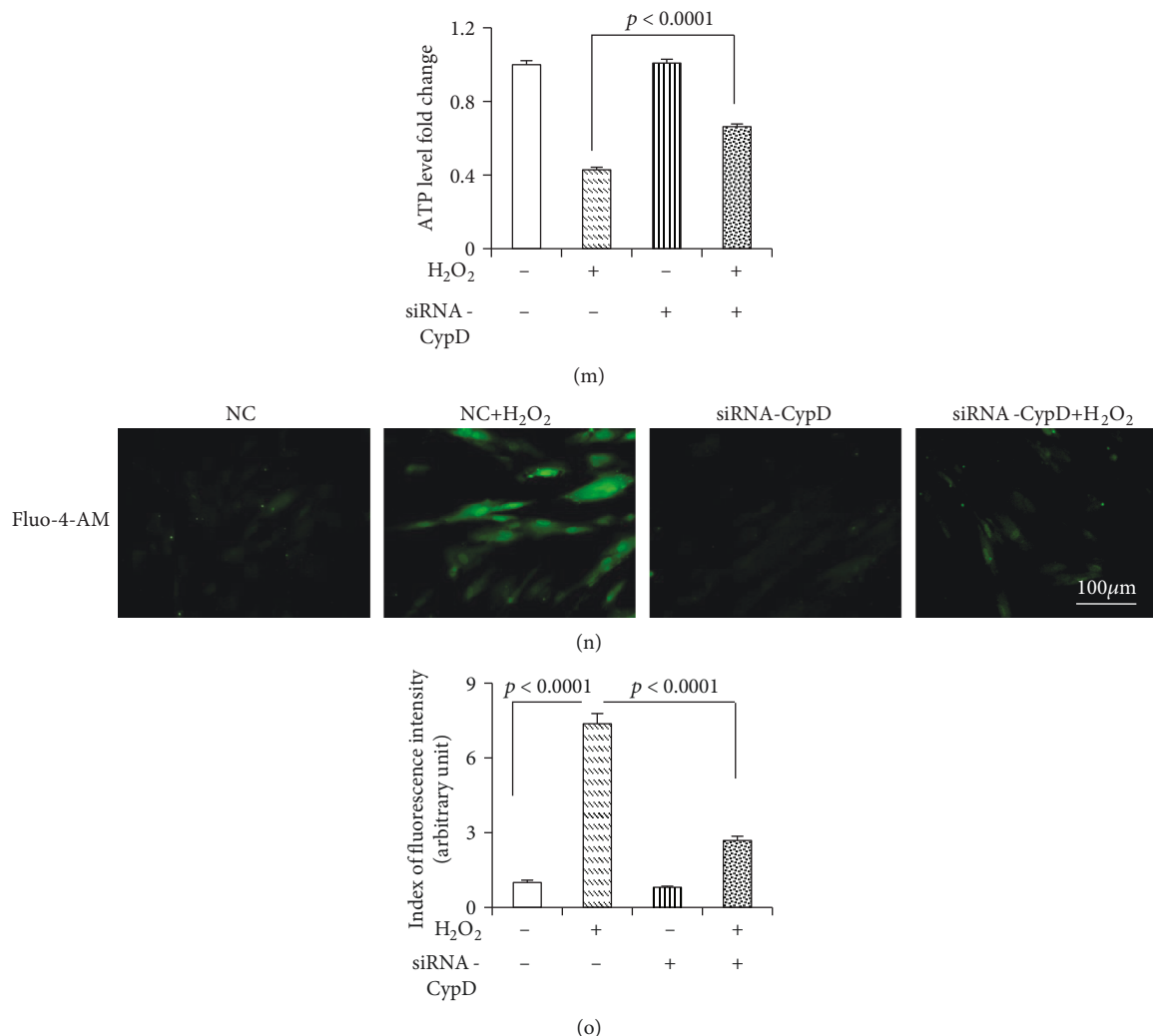


FIGURE 4: CypD siRNA attenuated H₂O₂-induced cell death and mitochondrial dysfunction in the HDPCs. (a) Representative immunoreactive bands for CypD in the HDPCs with CypD siRNA (+) or NC (-) treatment at a final concentration of 50 nM for 48 h. Quantification of immunoreactive bands for CypD (b) relative to β -actin. (c) Cell viability determined by MTT reduction in the HDPCs in the presence of H₂O₂ with or without siRNA-PPIF. (d) TUNEL staining and (e) assay after siRNA-PPIF treatment. (f) Representative immunoreactive bands for Bax, Bcl-2, and CypD in the HDPCs with (+) or without (-) siRNA-PPIF treatment in the presence of H₂O₂ (+) or culture medium (-). Quantification of immunoreactive bands for Bax (g), Bcl-2 (h), and CypD (i) relative to β -actin. Representative images showing MitoSOX staining (j) and quantification (k) in the indicated groups. Representative images showing TMRM staining (l) and quantification (m) in the indicated groups. ATP (n) in the indicated groups. Representative images showing Fluo-4-AM staining (o) and quantification (p) in the indicated groups. HDPCs were treated for 24 h with H₂O₂ (250 μ M) (+) or culture medium (-) in the CypD siRNA group (+) or the NC group (-). Data represent the mean values \pm SD of three independent experiments.

HDPC death. A mitochondria-specific antioxidant, mitoquinone, has been proved to be effective in ameliorating OS-related diseases [18, 34]. It would be highly interesting to apply these agents in this HDPC injury model to confirm the role of mitochondrial OS and dysfunction in OS-induced HDPC death in the future study.

The mPTP, initiated by severe OS and cytosolic Ca²⁺ overload, is a key regulator in cell death [35, 36]. The opening of mPTP leads to rapid loss of MMP (which abolishes all MMP-dependent mitochondrial activities, including ATP synthesis), osmotic breakdown of mitochondrial membranes, and structural breakdown of the organelle, eventually causing the regulated cell death [13, 35, 36]. In this study, OS

was shown to lead to mPTP opening in HDPCs, as reflected by MMP dissipation and Ca²⁺ disorder, which would result in the increase in mtROS and energy failure (decrease in ATP). A similar pattern was also found in H₂O₂-treated RAW264.7 macrophages [37]. NAC successfully inhibited mPTP opening and rescued mitochondrial dysfunction, thereby attenuating HDPC death. Taken together, these results showed that (i) mPTP opening was implicated in HDPC death and (ii) OS was the major inducer of mPTP opening-induced death in the H₂O₂-treated HDPCs.

In our study, we mainly focused on the mPTP opening-induced cell death. Other than CypD, several proteins including the voltage-dependent anion channel (VDAC), adenine

nucleotide translocator (ANT), and inorganic phosphate carrier (PHC) are supposed to be the putative modulators of mPTP [35]. However, previous studies using robust genetic tools confirm that CypD is the only vital protein *in vivo* required for mPTP opening [14, 38]. The knockout of VDAC, ANT, or PHC fails to protect cells from the mPTP opening [39–42]. In addition, mitochondrial F_1F_0 ATPase is also demonstrated to be involved in mPTP formation [43]. However, this conclusion is challenged by further reports by calculating their ion conductance and selectivity and especially the persistence of the mitochondrial permeability transition in the absence of the *c*-subunit of human F_1F_0 ATPase [44–46]. A very recent study largely gives structural explanation for the abovementioned inconsistent phenomena [47]. This study shows that there is no conventional mPTP formation but a CsA-sensitive channel when *c*-subunit is knocked out, still contributing to the depolarization of the inner mitochondrial membrane [47]. Further evidence will help to confirm the role of F_1F_0 ATPase in mPTP. In this study, we mainly focused on CypD and hypothesized that CypD was a key molecule in the H_2O_2 -induced HDPC death. As expected, CsA and CypD siRNA inhibited the H_2O_2 -induced mPTP opening, attenuated mitochondrial dysfunction, and prevented the death of HDPCs. Moreover, our results found that the CypD expression level significantly increased in the H_2O_2 -treated HDPCs. This was consistent with a previous study showing that the increased expression of CypD contributed to the oxidative injuries in endothelial cells induced by a thyroid-stimulating hormone [16]. An *in vivo* study also confirmed that OS-induced cell death was mediated by CypD overexpression in the fibroblasts of patients with X-linked adrenoleukodystrophy [48]. Nonetheless, the overexpression of CypD was not always indispensable in its mediated cell death [49]. CypD may mediate the mPTP opening through three main mechanisms: (1) physical relationships with pore components [50], (2) ANT-independent pore formation [39], and (3) mitochondrial crista remodeling on the complete discharge of cytochrome C [51]. Consequently, CypD-mediated mPTP opening, especially in response to OS and Ca^{2+} overload, can be attenuated by either CypD genetic deletion [14], CypD peptidyl-prolyl *cis*- or *trans*-isomerase activity inhibition, or CsA molecular conformation [39]. All findings underscore a vital role of CypD-driven mPTP opening in OS-induced mitochondrial dysfunction and cell death of HDPCs. However, we only analyzed the role of CypD in H_2O_2 -induced toxicity in dental pulp *in vitro*, and *in vivo* evidence should be achieved to corroborate the role of CypD.

The upstream molecules regulating CypD remain largely unknown in the H_2O_2 -induced death of HDPCs. A previous study indicates that gallic acid potentiates the extracellular signal-regulated kinase (ERK) phosphorylation, leading to a decrease in CypD expression, which contributes to the neuroprotective effect on cerebral ischemia/reperfusion injury [52]. Some studies also show that an increased thyroid-stimulating hormone can activate CypD by inhibiting sirtuin-3 (SIRT3) in endothelial cells [16]. Therefore, the search for vital upstream regulators of CypD in HDPCs, such as ERK, SIRT3, or p53, needs to be further explored in the future study.

5. Conclusions

Our data offered new insights into the role of CypD-mediated mitochondrial dysfunction in the H_2O_2 -induced HDPC death (Supplementary Figure 2) and the possible usage of the CypD inhibitor in the clinical treatment of dental pulp injury in the future.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Shengbin Huang, Bingbing Zheng, and Xing Jin contributed equally to this work.

Acknowledgments

This work was supported by the Natural Science Foundation of China (81870757, 81500817, and 81870777), Zhejiang Provincial Natural Science Foundation of China (LY16H140005), Health Science and Technology Project of Zhejiang Province (2016KYB184), and Wenzhou Public Technical Research Medical Program (2015Y0507).

Supplementary Materials

Figure S1: characterization of HDPCs. Figure S2: working hypothesis. (*Supplementary Materials*)

References

- [1] C. Hahn and F. Liewehr, "Relationships between caries bacteria, host responses, and clinical signs and symptoms of pulpitis," *Journal of Endodontics*, vol. 33, no. 3, pp. 213–219, 2007.
- [2] L. T. A. Cintra, F. Benetti, A. C. da Silva Facundo et al., "The number of bleaching sessions influences pulp tissue damage in rat teeth," *Journal of Endodontics*, vol. 39, no. 12, pp. 1576–1580, 2013.
- [3] S. Krifka, G. Spagnuolo, G. Schmalz, and H. Schweikl, "A review of adaptive mechanisms in cell responses towards oxidative stress caused by dental resin monomers," *Biomaterials*, vol. 34, no. 19, pp. 4555–4563, 2013.
- [4] H. Schweikl, W. Buchalla, and S. Krifka, "Cell responses to cariogenic microorganisms and dental resin materials-crosstalk at the dentin-pulp interface?," *Dental Materials*, vol. 33, no. 5, pp. 514–524, 2017.
- [5] F. Benetti, J. E. Gomes-Filho, L. L. Ferreira et al., "Hydrogen peroxide induces cell proliferation and apoptosis in pulp of rats after dental bleaching *in vivo*: effects of the dental bleaching in pulp," *Archives of Oral Biology*, vol. 81, pp. 103–109, 2017.

- [6] S. Huang, Y. Wang, X. Gan et al., "Drp1-mediated mitochondrial abnormalities link to synaptic injury in diabetes model," *Diabetes*, vol. 64, no. 5, pp. 1728–1742, 2015.
- [7] M.-C. Chang, L.-I. Chen, C.-P. Chan et al., "The role of reactive oxygen species and hemeoxygenase-1 expression in the cytotoxicity, cell cycle alteration and apoptosis of dental pulp cells induced by bisGMA," *Biomaterials*, vol. 31, no. 32, pp. 8164–8171, 2010.
- [8] I. L. Chapple, "Reactive oxygen species and antioxidants in inflammatory diseases," *Journal of Clinical Periodontology*, vol. 24, no. 5, pp. 287–296, 1997.
- [9] Y. Jiao, S. Ma, Y. Wang et al., "N-Acetyl cysteine depletes reactive oxygen species and prevents dental monomer-induced intrinsic mitochondrial apoptosis in vitro in human dental pulp cells," *PLoS One*, vol. 11, no. 1, article e0147858, 2016.
- [10] J. S. Takanche, Y.-H. Lee, J.-S. Kim et al., "Anti-inflammatory and antioxidant properties of schisandrin C promote mitochondrial biogenesis in human dental pulp cells," *International Endodontic Journal*, vol. 51, no. 4, pp. 438–447, 2018.
- [11] A. V. Vaseva, N. D. Marchenko, K. Ji, S. E. Tsirka, S. Holzmann, and U. M. Moll, "p53 opens the mitochondrial permeability transition pore to trigger necrosis," *Cell*, vol. 149, no. 7, pp. 1536–1548, 2012.
- [12] K. Santosh, S. L. Mehta, G. Z. Milledge, X. Huang, H. Li, and P. A. Li, "Ubisol-q10 prevents glutamate-induced cell death by blocking mitochondrial fragmentation and permeability transition pore opening," *International Journal of Biological Sciences*, vol. 12, no. 6, pp. 688–700, 2016.
- [13] P. Bernardi, A. Rasola, M. Forte, and G. Lippe, "The mitochondrial permeability transition pore: channel formation by F-ATP synthase, integration in signal transduction, and role in pathophysiology," *Physiological Reviews*, vol. 95, no. 4, pp. 1111–1155, 2015.
- [14] C. P. Baines, R. A. Kaiser, N. H. Purcell et al., "Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death," *Nature*, vol. 434, no. 7033, pp. 658–662, 2005.
- [15] D. B. Zorov, M. Juhaszova, and S. J. Sollott, "Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release," *Physiological Reviews*, vol. 94, no. 3, pp. 909–950, 2014.
- [16] X. Liu, H. Du, Q. Chai et al., "Blocking mitochondrial cyclophilin D ameliorates TSH-impaired defensive barrier of artery," *Redox Biology*, vol. 15, pp. 418–434, 2018.
- [17] P. Dai, Y. Mao, X. Sun et al., "Attenuation of oxidative stress-induced osteoblast apoptosis by curcumin is associated with preservation of mitochondrial functions and increased Akt-GSK3 β signaling," *Cellular Physiology and Biochemistry*, vol. 41, no. 2, pp. 661–677, 2017.
- [18] Y. X. Mao, W. J. Cai, X. Y. Sun et al., "Rage-dependent mitochondria pathway: a novel target of silibinin against apoptosis of osteoblastic cells induced by advanced glycation end products," *Cell Death & Disease*, vol. 9, no. 6, p. 674, 2018.
- [19] N. Lee, Y. Lee, G. Bhattari et al., "Reactive oxygen species removal activity of davallialactone reduces lipopolysaccharide-induced pulpal inflammation through inhibition of the extracellular signal-regulated kinase 1/2 and nuclear factor kappa B pathway," *Journal of Endodontics*, vol. 37, no. 4, pp. 491–495, 2011.
- [20] Y. He, L. Zhou, Z. Fan, S. Liu, and W. Fang, "Palmitic acid, but not high-glucose, induced myocardial apoptosis is alleviated by N-acetylcysteine due to attenuated mitochondrial-derived ROS accumulation-induced endoplasmic reticulum stress," *Cell Death & Disease*, vol. 9, no. 5, p. 568, 2018.
- [21] S. Wilson and A. Keenan, "Role of hemin in the modulation of H₂O₂-mediated endothelial cell injury," *Vascular Pharmacology*, vol. 40, no. 2, pp. 109–118, 2003.
- [22] L. Galluzzi, I. Vitale, S. A. Aaronson et al., "Molecular mechanisms of cell death: recommendations of the nomenclature committee on cell death 2018," *Cell Death & Differentiation*, vol. 25, no. 3, pp. 486–541, 2018.
- [23] T. Miyashita and J. Reed, "Tumor suppressor p53 is a direct transcriptional activator of the human bax gene," *Cell*, vol. 80, no. 2, pp. 293–299, 1995.
- [24] A. Gross, J. Jockel, M. C. Wei, and S. J. Korsmeyer, "Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis," *The EMBO Journal*, vol. 17, no. 14, pp. 3878–3885, 1998.
- [25] R. Salvador-Gallego, M. Mund, K. Cosentino et al., "Bax assembly into rings and arcs in apoptotic mitochondria is linked to membrane pores," *The EMBO Journal*, vol. 35, no. 4, pp. 389–401, 2016.
- [26] S. Aluvila, T. Mandal, E. Hustedt, P. Fajer, J. Y. Choe, and K. J. Oh, "Organization of the mitochondrial apoptotic BAK pore: oligomerization of the BAK homodimers," *Journal of Biological Chemistry*, vol. 289, no. 5, pp. 2537–2551, 2014.
- [27] L. A. Gillies, H. Du, B. Peters, C. M. Knudson, D. D. Newmeyer, and T. Kuwana, "Visual and functional demonstration of growing Bax-induced pores in mitochondrial outer membranes," *Molecular Biology of the Cell*, vol. 26, no. 2, pp. 339–349, 2015.
- [28] S. Cory, D. C. S. Huang, and J. M. Adams, "The bcl-2 family: roles in cell survival and oncogenesis," *Oncogene*, vol. 22, no. 53, pp. 8590–8607, 2003.
- [29] P. Czabotar, G. Lessene, A. Strasser, and J. M. Adams, "Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy," *Nature Reviews Molecular Cell Biology*, vol. 15, no. 1, pp. 49–63, 2014.
- [30] H. Chen, M. Kanai, A. Inoue-Yamauchi et al., "An interconnected hierarchical model of cell death regulation by the BCL-2 family," *Nature Cell Biology*, vol. 17, no. 10, pp. 1270–1281, 2015.
- [31] X. Luo, H. Zhang, Y. Duan, and G. Chen, "Protective effects of radish (*Raphanus sativus* L.) leaves extract against hydrogen peroxide-induced oxidative damage in human fetal lung fibroblast (MRC-5) cells," *Biomedicine & Pharmacotherapy*, vol. 103, pp. 406–414, 2018.
- [32] W. Sun, J. Yu, H. Gao et al., "Inhibition of lung cancer by 2-methoxy-6-acetyl-7-methyljuglone through induction of necroptosis by targeting receptor-interacting protein 1," *Antioxidants & Redox Signaling*, 2018, In press.
- [33] K. Rohde, L. Kleinesudeik, S. Roesler et al., "A Bak-dependent mitochondrial amplification step contributes to Smac mimetic/gluococorticoid-induced necroptosis," *Cell Death & Differentiation*, vol. 24, no. 1, pp. 83–97, 2017.
- [34] X. Sun, Y. Mao, P. Dai et al., "Mitochondrial dysfunction is involved in the aggravation of periodontitis by diabetes," *Journal of Clinical Periodontology*, vol. 44, no. 5, pp. 463–471, 2017.
- [35] V. Izzo, J. M. Bravo-San Pedro, V. Sica, G. Kroemer, and L. Galluzzi, "Mitochondrial permeability transition: new findings and persisting uncertainties," *Trends in Cell Biology*, vol. 26, no. 9, pp. 655–667, 2016.

- [36] T. Vanden Berghe, A. Linkermann, S. Jouan Lanhouet, H. Walczak, and P. Vandenabeele, "Regulated necrosis: the expanding network of non-apoptotic cell death pathways," *Nature Reviews Molecular Cell Biology*, vol. 15, no. 2, pp. 135–147, 2014.
- [37] X. Zhang, X. Sun, C. Wu et al., "Heme oxygenase-1 induction by methylene blue protects RAW264.7 cells from hydrogen peroxide-induced injury," *Biochemical Pharmacology*, vol. 148, pp. 265–277, 2018.
- [38] T. Nakagawa, S. Shimizu, T. Watanabe et al., "Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death," *Nature*, vol. 434, no. 7033, pp. 652–658, 2005.
- [39] J. E. Kokoszka, K. G. Waymire, S. E. Levy et al., "The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore," *Nature*, vol. 427, no. 6973, pp. 461–465, 2004.
- [40] C. P. Baines, R. A. Kaiser, T. Sheiko, W. J. Craigen, and J. D. Molkenin, "Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death," *Nature Cell Biology*, vol. 9, no. 5, pp. 550–555, 2007.
- [41] J. Q. Kwong, J. Davis, C. P. Baines et al., "Genetic deletion of the mitochondrial phosphate carrier desensitizes the mitochondrial permeability transition pore and causes cardiomyopathy," *Cell Death & Differentiation*, vol. 21, no. 8, pp. 1209–1217, 2014.
- [42] A. Richardson and A. Halestrap, "Quantification of active mitochondrial permeability transition pores using GNX-4975 inhibitor titrations provides insights into molecular identity," *Biochemical Journal*, vol. 473, no. 9, pp. 1129–1140, 2016.
- [43] G. Beutner, E. Lazrove, S. Sacchetti et al., "An uncoupling channel within the c-subunit ring of the F_1F_0 ATP synthase is the mitochondrial permeability transition pore," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 29, pp. 10580–10585, 2014.
- [44] W. Zhou, F. Marinelli, C. Nief, and J. D. Faraldo-Gómez, "Atomistic simulations indicate the c-subunit ring of the F_1F_0 ATP synthase is not the mitochondrial permeability transition pore," *eLife*, vol. 6, 2017.
- [45] J. He, J. Carroll, S. Ding, I. M. Fearnley, and J. E. Walker, "Permeability transition in human mitochondria persists in the absence of peripheral stalk subunits of ATP synthase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, no. 34, pp. 9086–9091, 2017.
- [46] J. He, H. Ford, J. Carroll, S. Ding, I. M. Fearnley, and J. E. Walker, "Persistence of the mitochondrial permeability transition in the absence of subunit c of human ATP synthase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, no. 13, pp. 3409–3414, 2017.
- [47] M. A. Neginskaya, M. E. Solesio, E. V. Berezhnaya et al., "ATP synthase c-subunit-deficient mitochondria have a small cyclosporine A-sensitive channel, but lack the permeability transition pore," *Cell Reports*, vol. 26, no. 1, pp. 11–17.e2, 2019.
- [48] J. Lopez-Erauskin, J. Galino, P. Bianchi et al., "Oxidative stress modulates mitochondrial failure and cyclophilin D function in X-linked adrenoleukodystrophy," *Brain*, vol. 135, no. 12, pp. 3584–3598, 2012.
- [49] Y. Zhen, G. Wang, L. Zhu et al., "P53 dependent mitochondrial permeability transition pore opening is required for dexamethasone-induced death of osteoblasts," *Journal of Cellular Physiology*, vol. 229, no. 10, pp. 1475–1483, 2014.
- [50] D. R. Green and G. Kroemer, "The pathophysiology of mitochondrial cell death," *Science*, vol. 305, no. 5684, pp. 626–629, 2004.
- [51] L. Scorrano, M. Ashiya, K. Buttle et al., "A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis," *Developmental Cell*, vol. 2, no. 1, pp. 55–67, 2002.
- [52] J. Sun, D.-D. Ren, J.-Y. Wan et al., "Desensitizing mitochondrial permeability transition by ERK-cyclophilin D axis contributes to the neuroprotective effect of gallic acid against cerebral ischemia/reperfusion injury," *Frontiers in Pharmacology*, vol. 8, p. 184, 2017.



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