

RESEARCH ARTICLE

Effects of Camphorquinone on Cytotoxicity, Cell Cycle Regulation and Prostaglandin E₂ Production of Dental Pulp Cells: Role of ROS, ATM/Chk2, MEK/ERK and Hemeoxygenase-1

Mei-Chi Chang¹, Li-Deh Lin^{2,3}, Min-Tsz Wu^{2,3}, Chiu-Po Chan⁴, Hsiao-Hua Chang^{2,3}, Ming-Shu Lee^{2,3}, Tzu-Ying Sun^{2,3}, Po-Yuan Jeng⁵, Sin-Yuet Yeung⁴, Hsueh-Jen Lin⁶, Jjiang-Huei Jeng^{2,3*}

1 Biomedical Science Team, Chang Gung University of Science and Technology, Kwei-Shan, Taoyuan City, Taiwan, **2** Laboratory of Dental Pharmacology, Toxicology & Material Biocompatibility, Graduate Institute of Clinical Dentistry, and National Taiwan University Medical College, Taipei, Taiwan, **3** Department of Dentistry, National Taiwan University Hospital, Taipei, Taiwan, **4** Department of Dentistry, Chang Gung Memorial Hospital, Taipei, Taiwan, **5** School of Dentistry, University of Cardenal Herrera, CEU, Valencia, Spain, **6** Department of Dentistry, Show Chwan Memorial Hospital, Chang-Hua, Taiwan

* jhjeng@ntu.edu.tw; benson86778231@yahoo.com.tw



OPEN ACCESS

Citation: Chang M-C, Lin L-D, Wu M-T, Chan C-P, Chang H-H, Lee M-S, et al. (2015) Effects of Camphorquinone on Cytotoxicity, Cell Cycle Regulation and Prostaglandin E₂ Production of Dental Pulp Cells: Role of ROS, ATM/Chk2, MEK/ERK and Hemeoxygenase-1. PLoS ONE 10(12): e0143663. doi:10.1371/journal.pone.0143663

Editor: Ying-Jan Wang, National Cheng Kung University, TAIWAN

Received: August 15, 2015

Accepted: November 6, 2015

Published: December 14, 2015

Copyright: © 2015 Chang et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper.

Funding: This study is supported by grants from Ministry of Science and Technology (NSC102-2314-B-255-003-MY2, NSC102-2628-B-255-001-MY3, NSC101-2320-B-255-002, NSC-100-2314-B-002-094, NSC-101-2320-B-255-002, and NSC102-2314-B002-051-MY2), National Taiwan University Hospital (NTUH) and Chang Gung Memorial Hospital (CMRPF1C0011, CMRPF1C0012, NMRPF3C0091,

Abstract

Camphorquinone (CQ) is a popularly-used photosensitizer in composite resin restoration. In this study, the effects of CQ on cytotoxicity and inflammation-related genes and proteins expression of pulp cells were investigated. The role of reactive oxygen species (ROS), ATM/Chk2/p53 and hemeoxygenase-1 (HO-1) and MEK/ERK signaling was also evaluated. We found that ROS and free radicals may play important role in CQ toxicity. CQ (1 and 2 mM) decreased the viability of pulp cells to about 70% and 50% of control, respectively. CQ also induced G₂/M cell cycle arrest and apoptosis of pulp cells. The expression of type I collagen, cdc2, cyclin B, and cdc25C was inhibited, while p21, HO-1 and cyclooxygenase-2 (COX-2) were stimulated by CQ. CQ also activated ATM, Chk2, and p53 phosphorylation and GADD45α expression. Besides, exposure to CQ increased cellular ROS level and 8-isoprostane production. CQ also stimulated COX-2 expression and PGE₂ production of pulp cells. The reduction of cell viability caused by CQ can be attenuated by N-acetyl-L-cysteine (NAC), catalase and superoxide dismutase (SOD), but can be promoted by Zinc protoporphyrin (ZnPP). CQ stimulated ERK1/2 phosphorylation, and U0126 prevented the CQ-induced COX-2 expression and prostaglandin E₂ (PGE₂) production. These results indicate that CQ may cause cytotoxicity, cell cycle arrest, apoptosis, and PGE₂ production of pulp cells. These events could be due to stimulation of ROS and 8-isoprostane production, ATM/Chk2/p53 signaling, HO-1, COX-2 and p21 expression, as well as the inhibition of cdc2, cdc25C and cyclin B1. These results are important for understanding the role of ROS in pathogenesis of pulp necrosis and pulpal inflammation after clinical composite resin filling.

NMRPF3C0061), and National Taiwan University Hospital (NTUH).

Competing Interests: The authors have declared that no competing interests exist.

Introduction

In dentistry, resin composites are widely used as restorative materials because of their ease of handling and esthetic enhancement. The commonly used oligomers and monomers in organic polymer matrix of resin composites belong to dimethacrylates, which contain reactive carbon double bonds. They undergo free-radical polymerization that is a kind of addition polymerization, and polymerization initiators are contained to produce free radicals for initiating the reaction. The polymerization initiators used for light-cured resin composites usually consist of a photosensitizer, primarily camphorquinone (CQ), and a reducing agent which is often a tertiary amine such as dimethylaminoethyl methacrylate (DMAEMA) or dimethyl-para-toluidine (DMPT) [1].

The concentration of CQ in the resin phase usually ranges from 0.17% to 1.03% w/w [2]. CQ has two carbonyl groups with non-bonding electrons, and the absorption spectrum of it is relatively broad between 400 and 550 nm in the blue region of visible light, with the maximum at 468 nm. CQ produces a pair of free radicals through proton abstraction [3]. The monomer-polymer conversion rate of resin composites varies approximately from 35% to 77% [4]. The residual monomers and additives are free to diffuse out from the cured materials. They may be released into surrounding tissues, and may have potential toxic effects. CQ was identified as one of the main released components in extracts of resin-based materials [4,5].

Initiating radicals may indiscriminately react with molecular oxygen forming reactive oxygen species (ROS), which may potentially cause oxidative damage to the cells' macromolecules. Generally, CQ reveals a moderate cytotoxic effect compared to other photoinitiators and most resin (co)monomers [6]. Studies on CQ are limited comparing to those on resin (co)monomers. Masuki *et al.* reported a statistically significant finding of growth inhibition and G₀/G₁ cell cycle arrest in human gingival fibroblasts (HGF) treated with 1 and 5 mM CQ for 24 hours. They also noted that exposure to 5 mM CQ increased the numbers of apoptotic/necrotic cells [1]. Engelmann *et al.* found that at concentrations higher than 1 mM, CQ caused a significant concentration-dependent increase of intracellular ROS in human pulp fibroblasts (HPF) within 90 minutes of exposure. Moreover, the ROS increase was associated with a moderate decrease of glutathione (GSH), the most important intracellular ROS-scavenger, after treatment by 5 mM CQ for 90 minutes [7]. Volk *et al.* treated HGF with CQ or CQ in combination with 0.5 mM N-acetylcysteine (NAC), a ROS-scavenger, for 3 hours. The data showed that at concentrations higher than 1.25 mM, CQ caused a significant concentration-dependent increase of intracellular ROS, which was only associated with a moderate glutathione (GSH) decrease at the highest concentration of 2.5 mM CQ. They also found that NAC reduced CQ-induced ROS formation [8]. However, influences of CQ on cell cycle and cell death in human dental pulp cells are not available in the literature. In addition, the changes of the related genes and proteins expression are still not clear nowadays. Hemeoxygenase (HO) is the rate-limiting enzyme of microsomal heme degradation pathway, and biliverdin, one of the final products, is further converted to bilirubin. HO has been suggested to function as a defense system against oxidative stress, since biliverdin or bilirubin produced locally in the body may act as physiological antioxidants. HO-1 is an inducible isoform in response to stress such as oxidative stress, hypoxia, heavy metals, cytokines, and so forth [9]. However, the role of HO-1 in regulation of CQ toxicity is not clear.

The cell cycle was divided into four distinct phases: G₁, S, G₂, and M. The transition from one cell cycle phase to another depends on a series of sequential events. The key regulatory proteins are the cyclin-dependent kinases (CDK) and their activating proteins, the cyclins. Different cyclin/CDK complexes are assembled and activated at different points of the cell cycle. CDK activity can be counteracted by cell cycle inhibitory proteins such as various CDK

inhibitors (CKI). There are two different classes of CKI. The INK4 family includes p15, p16, p18, and p19, which specifically target CDK4 and CDK6. As for the Cip/Kip family, its family members consist of p21, p27, and p57. They inhibit a wide spectrum of CDKs [10]. Since cells are constantly under the attack of endogenous and exogenous damage, cells have evolved general mechanisms called checkpoints that monitor and confirm the successful completion of cell cycle events [11]. DNA damage may activate the checkpoint transducing kinases such as Ataxia telangiectasia mutated (ATM)/ ataxia-telangiectasia and Rad3 related (ATR) and checkpoint kinase 1 and 2 (Chk1/Chk2). Then, at the beginning of DNA damage, the acute and transient cell cycle delay is activated. Its downstream targets include *cdc25A* for G₁/S, Nbs1 as well as SMC1 for intra-S, and *cdc25C* for G₂/M phase. On the other hand, the p53-dependent delay and sustain of cell cycle arrest is slow-operating. Its targets are p21 for G₁/S, and p21, 14-3-3 σ as well as growth arrest and DNA damage inducible protein—GADD45 for G₂/M phase [12]. Generally there are two types of cell death, namely, apoptosis and necrosis. When cytotoxic stimuli are intense, cells may get away from the cell cycle and undergo a programmed cell death called apoptosis. By contrast, necrosis is a kind of “cell murder” that follows the exposure of cells to a gross injury [1].

Clinically, some of the teeth may develop pulpitis and pulp necrosis after composite resin restoration. This is partly due to the toxicity of resin monomers or photo-initiator such as CQ. The purpose of this study is to investigate the influences of CQ with different concentrations on cytotoxicity to human dental pulp cells, including morphological changes, cell proliferation, cell cycle progression, cell death pattern and prostanooids production. Then, the expressions of cyclooxygenase-2 (COX-2), cell cycle regulation and apoptosis related genes and proteins under the treatment of CQ are evaluated. Besides, the roles of ROS, ATM/Chk2, HO-1 and MEK/extracellular signal-regulated kinase (ERK) in the CQ-induced cell changes are also evaluated.

Materials and Methods

Materials

CQ, dimethylsulfoxide (DMSO), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), propidium iodide (PI), N-acetylcysteine (NAC), catalase, superoxide dismutase (SOD) and Zinc protoporphyrin (ZnPP) were obtained from Sigma (Sigma Chemical Company, St Louis, MO, USA). The cell culture biological such as Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were bought from Gibco (Life Technologies, Grand Island, NY, USA). Annexin V and reagents for flow cytometry were obtained from Becton Dickinson (Worldwide Inc., San-Jose, CA). RNA isolation kit and NucleoSpin RNA II were purchased from Macherey-Nagel (Macherey-Nagel Inc, Easton, PA, USA). The SuperScriptTM III First-Strain DNA Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) was from Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA). The luminol reagents for western blotting and the primary antibodies Type I collagen, p-ERK, GAPDH, COX-2, HO-1, *cdc2*, cyclin B1, *cdc25C*, p-ATM, p-Chk2, p-p53, GADD45 α were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and p21 was from GeneTex (Irvine, California, USA). Enzyme-linked immunosorbant assay (ELISA) kits for PGE₂ and 8-isoprostane were from Cayman Chemical Company (Ann Arbor, MI, USA).

Culture of Human Dental Pulp Cells

By the approval of Ethics Committee, National Taiwan University Hospital, three strains of human dental pulp cells were cultured from extracted premolars of patients under 25 years old

with written informed consents by the patients or next of kin. Briefly, human dental pulp tissues were taken from caries- and periodontitis-free premolars extracted from three young donors (12–20 years old) for orthodontic purposes with proper written informed consent by the patients or next of kin on behalf of all minors enrolled in this study. Pulp cells were cultured by using a tissue explant technique [13–17]. Briefly a hammer was used to split the teeth in order to get the vital pulp tissues. Then a surgical knife was used to cut the dental pulp tissues into small pieces (about 1 mm³). The tissues were placed into 10-cm dishes and cultured by DMEM containing 10% FBS, 1x penicillin/streptomycin. When the growth of cells were about to reach the confluence, cells were detached from culture dishes by treatment with trypsin/ethylenediamine tetraacetic acid (EDTA) for subculture. The 3rd to 8th generations of the pulp cells were used in this study.

Cytotoxicity of CQ on Human Dental Pulp Cells

In brief, 2.5×10^5 human dental pulp cells were placed into 6-well culture plates with 2 ml of DMEM containing 10% FBS. After 24 hours of incubation, the culture medium was renewed by a fresh one containing 0.4% (v/v) DMSO (solvent) or various concentrations of CQ (0.1, 0.25, 0.5, 1, and 2 mM). After 24 hours of exposure, the morphological changes of human dental pulp cells were observed and photographed under a phase contrast microscope (Olympus IX 71, Olympus America Inc.). The culture medium was collected for analysis. Then the culture medium with MTT (0.5 mg/ml) was added and incubated for further 2 hours. Finally medium was decanted and the produced insoluble formazan was dissolved in 1 ml DMSO and read against blank (DMSO) at OD₅₄₀ using a Dynatech Microwell plate reader (Dynatech Labs Inc., Chantilly, VA, USA) for cytotoxicity measurement as before [13–18]. Cell viability was estimated using the following formula: (sample values of OD₅₄₀-blank value)/(NC value of OD₅₄₀-blank value)×100%. In some experiments, cells were pretreated with aspirin, NAC, catalase, SOD, ZnPP, or U0126 for 30 min and then CQ (final 2 mM) was added and co-incubated for 24 hours. Culture medium was collected for ELISA. Cytotoxicity was determined by MTT assay as above.

Flow Cytometric Analysis of Cell Cycle Distribution and Apoptosis

In short, 2.5×10^5 human dental pulp cells were incubated in each well of a 6-well plate in 2 ml of DMEM with 10% FBS. 24 hours later, the medium was replaced by a fresh one containing 0.4% v/v DMSO (NC) or different concentrations of CQ (0.1, 0.25, 0.5, 1 and 2 mM) and further cultured for 24 hours.

Cell Cycle Analysis: PI Staining Flow Cytometric Analysis

After exposure of pulp cells to CQ, both the attached and the floating cells were collected. They were resuspended and fixed in 70% ethanol at -20°C for 30 minutes. After centrifugation and washed with PBS, 350 µl of propidium iodide (PI, 40 µg/ml) was added for cell staining and then 2 µl of RNase A was added. After staining, the PI-elicited fluorescence of individual cells was determined by a FACS Calibur Flow Cytometer (Becton Dickinson, Worldwide Inc., San-Jose, CA, USA) [15,19,20]. Totally the PI fluorescence of 10000 cells was counted for each sample. The percentage of cells residing in sub-G₀/G₁ phase was measured using the CELL Quest program (Becton Dickinson, CA, USA), while the distribution of cells in G₀/G₁, S, and G₂/M phase were calculated with the ModFit LT program version 2.0 (Verity Software House, Inc., USA).

Cell Death Pattern Analysis by PI/Annexin V Flow Cytometric Analysis

Both the attached and floating cells were collected together. They were resuspended in 300 μ l of incubation buffer and stained with 8 μ l of PI (50 μ g/ml) and 4 μ l of Annexin V for about 30 minutes [15]. Later, the PI- and Annexin V-elicited fluorescence of individual cell was measured using a FACS Calibur Flow Cytometer (Becton Dickinson, Worldwide Inc., San-Jose, CA, USA). A total of 10000 cells were analyzed for each sample. The percentages of stained cells distributed in the lower left [LL: PI(-), Annexin V(-)], lower right [LR: PI(-), Annexin V (+)], upper left [UL: PI(+), Annexin V(-)], and upper right [UR: PI(+), Annexin V(+)] quadrants were determined using the CELL Quest program (Becton Dickinson, CA, USA).

Cellular ROS Production: Flow Cytometric Analysis of Cellular DCF Fluorescence and the Measurement of Medium 8-Isoprostane Level

Cells were exposed to different concentrations of CQ for 2.5 hours and then stained with DCFH-DA at the concentration of 10 μ M for further 30 minutes (total 3 hours). Then, both floating and attached cells were collected together. The cells were then re-suspended in 200 μ l of phosphate buffered saline (PBS) and the DCF-elicited fluorescence of individual cells was measured by using a FACS Calibur Flow Cytometer (Becton Dickinson, Worldwide Inc., San-Jose, CA, USA) [13,15,19]. A total of 10000 cells were analyzed for each sample. The mean of DCF fluorescence was determined by using the CELL Quest program (Becton Dickinson, CA, USA). The level of 8-isoprostane in culture medium was measured with ELISA kits.

Cellular PGE₂ and 8-Isoprostane Production

For measurement of PGE₂ production, pulp cells were exposed to CQ for 24 hours. Culture medium was collected for measuring the 8-isoprostane and PGE₂ production by ELISA. In some experiments, cells were pretreated with ZnPP, and U0126 for 30 min and then CQ (final 2 mM) was added and co-incubated for 24 hours. Culture medium was collected for ELISA analysis of PGE₂ production [13,21].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for Analysis of mRNA Expression

1.5 \times 10⁶ dental pulp cells were cultured in 10-cm dishes containing 10 ml of DMEM with 10% FBS. Twenty-four hours later, the culture medium was changed to a fresh one containing 0.4% v/v DMSO (NC) or various concentrations of CQ (0.1, 0.25, 0.5, 1 and 2 mM), and incubated for another 24 hours. Then, Total cellular RNA of dental pulp cells was isolated by using the RNA isolation kits. RT-PCR was used to evaluate the mRNA expression of human dental pulp cells.

Briefly denatured RNA (5 μ g) was reverse transcribed at 42°C for 90 minutes in a thermal cycler. The reaction mixture (45 μ l) contained 4 μ l of random primer (500 μ g/ml), 8 μ l of dNTP (2.5 mM), 4.5 μ l of 10x RT buffer, 1 μ l of RNase inhibitor (40 U/ μ l), 0.5 μ l of RT (21 U/ μ l), and double distilled water. The generated cDNA product was used for further PCR amplification in a reaction mixture comprising 5 μ l of 10x Super TAQ buffer, 4 μ l of 2.5 mM dNTP, 1 μ l of each specific primer, 0.2 μ l of Super TAQ enzyme (2 U/ μ l), and double distilled water. The specific primers were: cdc2: GGGGATTCAGAAATT GATCA and TGTCAGAAAGCTA CATCTTC (288 bp product), cdc25C: CCTGGTGAGAATTCGAAGACC and GCAGATGAA GTACACAT TGCATC (456 bp), cyclin B1: AAGAGCTTTAAACTTTGGTCTGGG and CTTTGTAAGTCC TTGATTTACCATG (317 bp), p21: GAGCGATGGAACCTCGA CTTTGTCACC and CTGAGACTAAGGCAGAAG ATGTAGAGCG (450 bp), HO-1: AAGA

TTGCCAGAAAGCCCTGGAC and CCAGAAAGCTGAG (399 bp), COX-2: TTCAAATGA GATTGTGGGAAAATTGCT-30 and 50-AGATCATCTCTGCCTGAGTATCTT (305 bp), and β -actin (BAC, 218 bp): AAGAGAGGCATCCTC ACCCT and TACATGGCTGGGGTGT TGAA as control [13,21, 22]. The amplification procedure for the target genes included 20–35 cycles of PCR, denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. This was followed by a final incubation at 72°C for 7 min. The PCR products were applied for 1.8% agarose gel electrophoresis, and DNA was stained with ethidium bromide for further photograph and presentation.

Western Blotting Analysis of Protein Expression

Briefly, 1.5×10^6 human dental pulp cells were seeded into 10-cm culture dishes with 10 ml of DMEM with 10% FBS. After 24 hours, the medium was replaced by a fresh one containing DMSO (vehicle control, NC) or different concentrations of CQ (0.1, 0.25, 0.5, 1, and 2 mM). After 24 hours, cells were washed with PBS and then disrupted in lysis buffer (10 mM Tris-HCl, pH 7; 140 mM sodium chloride; 3 mM magnesium chloride; 0.5% NP-40; 2 mM phenylmethylsulfonyl fluoride; 1% aprotinin; and 5 mM dithiothreitol) [13,23]. Then aliquots (20–50 μ g protein) of cell lysates were loaded to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blotted first with anti-human HO-1, cdc2, cdc25c, cyclin B1, COX-2, p-ERK1/ERK2 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibodies for 2-hr. This was followed by incubation with horseradish peroxidase-link secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hr. After washing the membrane with buffer, ECL reagents (Amersham) were added and the chemiluminescence was detected by exposure of membranes to Fuji films for 30 sec to 10 min. The intensity of GAPDH was used as control.

Immunofluorescent Microscope Observation of p-ATM, p-Chk2, p53 and Growth Arrest and DNA Damage-45 α (GADD-45 α) Protein Expression

In brief, 4×10^4 human dental pulp cells were seeded on the sterile coverslips in a 24-well plate in DMEM and 10% FBS. After 24 hours, they were exposed to different concentrations of CQ (0.25, 0.5, 1, 2, 3 mM) for further 24 hours. Medium was decanted, and cells were washed with PBS and fixed in 4% paraformaldehyde for 20 minutes. Cells were washed with PBS, permeabilized with 2% Triton X-100, incubated in 0.3% v/v H₂O₂ for 20 minutes. After washed with PBS, cells were blocked in 5% bovine serum albumin (BSA) for 1 hour and then incubated in primary antibodies (p-ATM, p-Chk2, p-p53 and GADD) (1:1000, v/v) at room temperature overnight. After PBS wash, cells were incubated in corresponding secondary antibody (FITC- or TRITC-conjugated) in the dark for 1 hour and counterstained with DAPI (1:1000) for 30 minutes. Finally the samples were mounted and observed/photographed under Olympus IX71 inverted microscope and DP Controller/Manager software (Olympus Corporation).

Statistical Analysis

All experiments were performed separately for at least 3 times. The means and standard errors (SE) of each experiment were calculated. The differences between experimental and control groups were analyzed by One-way ANOVA and post hoc Tukey test using the SPSS 10.0 software for windows. A p-value < 0.05 was considered to constitute a significant difference between the groups.

Results

Cytotoxicity of CQ on Human Dental Pulp Cells

Human dental pulp cells are spindle-shaped in appearance with extended cellular process when observed under a phase-contrast microscope (Fig 1A). After incubated with lower concentrations of CQ (0.1, 0.25, and 0.5 mM) for 24 hours, the morphology of cells was not altered markedly but the cell density seemed to be a little less than that of control group (data not shown). However, following exposure to higher concentrations of 1 mM and 2 mM CQ for 24 hours, the cells became much sparser in arrangement, smaller, retracted, and even rounded, especially in the CQ 2 mM group (Fig 1B and 1C). Cell proliferation was expressed as the percentage of cells proliferating in the presence of CQ relative to the cells proliferating in the absence of CQ (NC). As shown in Fig 1D, CQ inhibited the growth of human dental pulp cells in a dose-dependent manner. Cell viability was significantly reduced to about 70% and 50% of control under the treatment of 1 mM and 2 mM CQ ($p < 0.05$) for 24 hours.

Effects of CQ on Cell Cycle Progression of Pulp Cells

Human dental pulp cells treated with CQ demonstrated growth arrest. In untreated cells (NC), there were 69%, 17% and 14% of cells residing in G_0/G_1 , S and G_2/M phase of cell cycle

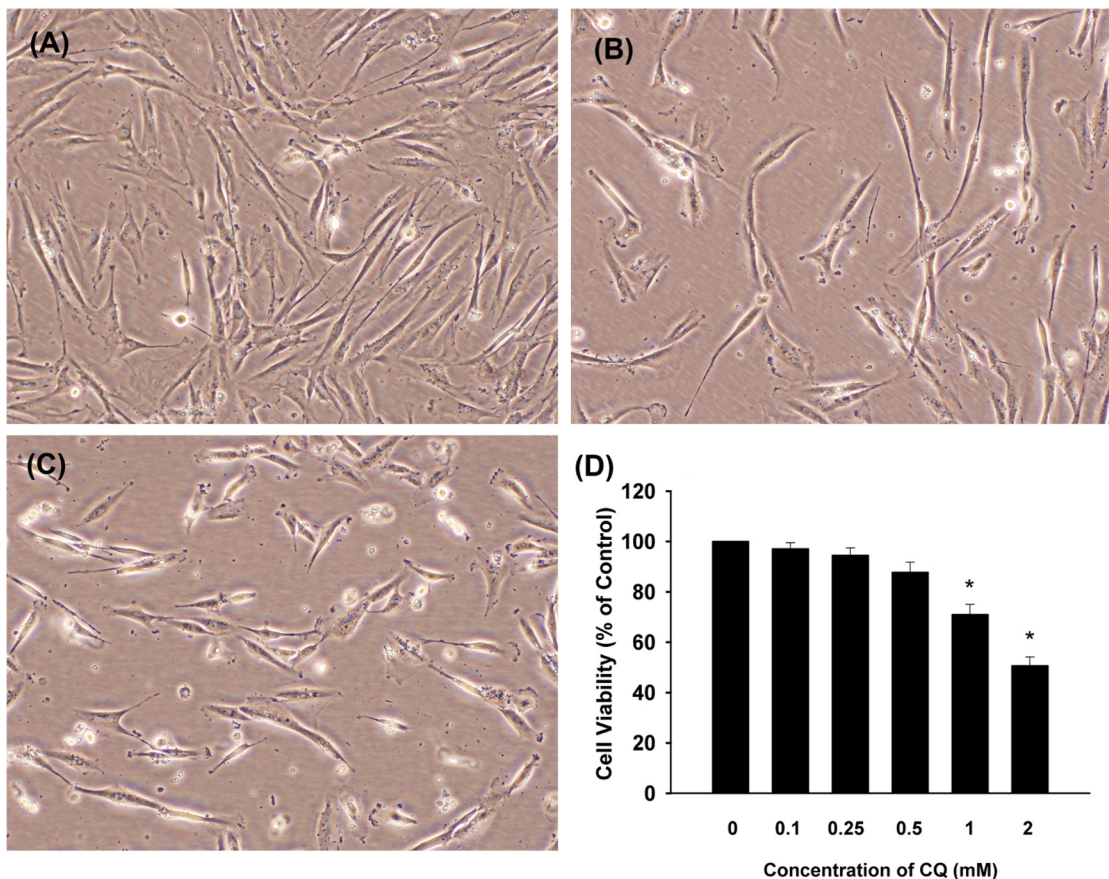


Fig 1. Morphology of human dental pulp cells after exposure to CQ for 24 hours. (A) Control (solvent), (B) 1 mM CQ, (C) 2 mM CQ, (100x, original magnification), (D) Quantitative cytotoxicity of CQ on dental pulp cells as analyzed by MTT assay. Results were expressed as % of control (as 100%). *denotes statistically significant difference ($p < 0.05$) when compared with solvent control group.

doi:10.1371/journal.pone.0143663.g001

respectively, with no obvious changes after incubation in 0.1–0.5 mM CQ. Exposure of pulp cells to 1 mM CQ led to a moderate increasing percentage of cells residing in G₂/M phase (22%, $p = 0.067$). However, following the exposure to 2 mM CQ for 24 hours, significant G₂/M cell cycle arrest was noted as revealed by increasing percentage of cells to 30% ($p < 0.05$) (Fig 2A). Besides, evident increase in sub-G₀/G₁ peak was noted in 1 mM and 2 mM ($p < 0.05$) CQ groups, indicating the possible induction of cellular apoptosis (Fig 2B). PI-annexin V dual staining flow cytometric analysis further found that there were 94.4%, 1.9%, 1.0%, and 2.7% of cells residing in LL, LR, UL, and UR quadrant respectively in control cells (NC). No marked changes after incubation in 0.1, 0.25, and 0.5 mM CQ for 24 hours. Exposure of pulp cells to 1 mM CQ led to an obvious increased percentage of cells in LR quadrant (pro-apoptotic, 4.8%, $p = 0.084$). A significant increase of cells in LR (5.2%, $p < 0.05$), UL (necrosis, 4.98%, $p < 0.05$) and UR (apoptotic, 8.21%, $p < 0.05$) quadrant was noted after the exposure of pulp cells to 2 mM CQ (Fig 2C and 2D).

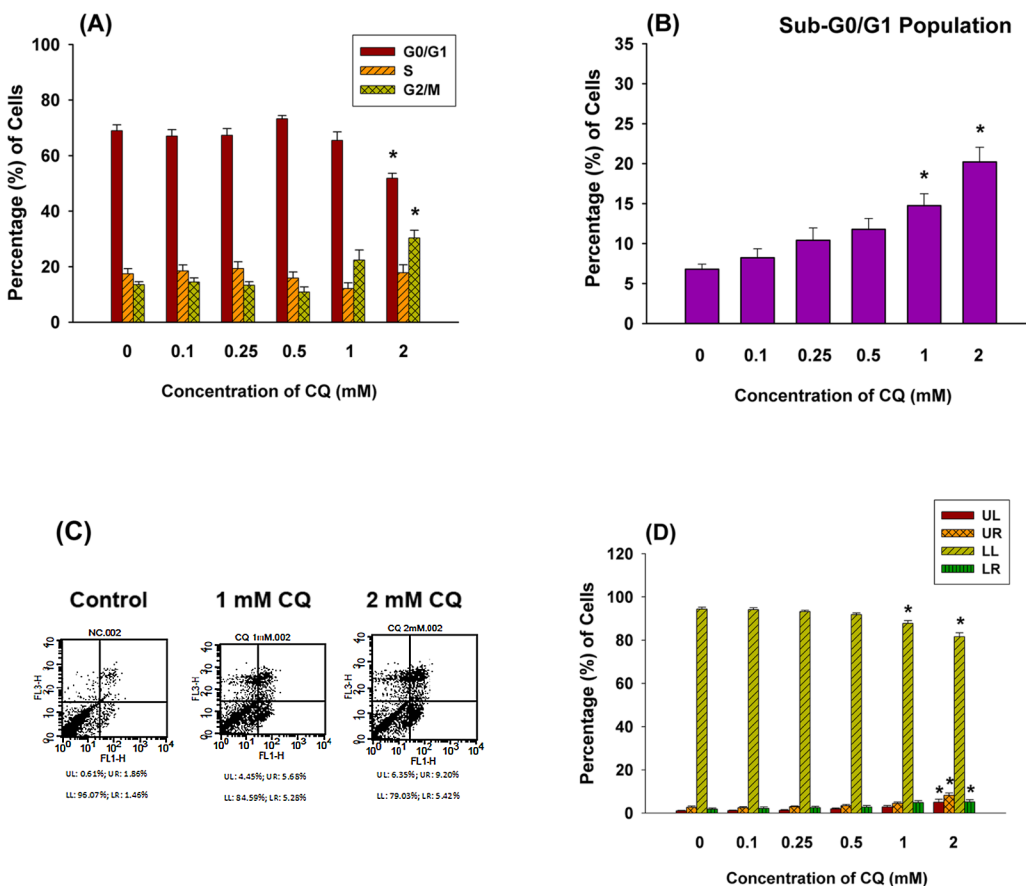


Fig 2. Effect of 24-hour exposure to CQ on the cell cycle progression and apoptosis of pulp cells. (A) Quantitative histogram representing the percentage of cells residing in each cell cycle phase ($n = 9$, Mean \pm SE, *denotes the presence statistically significant difference when compared with control ($p < 0.05$)). (B) Quantitative histogram representing the percentage of cells residing in sub-G₀/G₁ phase was shown ($n = 9$, Mean \pm SE, * denotes a p -value < 0.05). (C) Inducing the apoptosis of pulp cells by CQ as analyzed by PI/Annexin V dual staining flow cytometry. One representative PI/Annexin V flow cytometry profiles of pulp cells. Human dental pulp cells were exposed to CQ for 24 hours. In each plot, the lower left quadrant represents viable cells [LL: PI (-), Annexin V(-)], the lower right quadrant represents apoptotic cells [LR: PI(-), Annexin V(+)], the upper left quadrant represents necrotic cells [UL: PI(+), Annexin V(-)], and the upper right quadrant represents primary apoptotic, secondary necrotic cells [UR: PI(+), Annexin V(+)]. (D) Quantitative histogram of PI/Annexin V assay ($n = 5$, Mean \pm SE, *denotes a statistically significant difference when compared with solvent-treated control ($p < 0.05$)).

doi:10.1371/journal.pone.0143663.g002

mRNA and Protein Expression of Cell Cycle-Related Genes

Since exposure to CQ led to cell cycle arrest, further evaluation was done for the expression of cell cycle G2/M phase-related genes, including *cdc2*, *cdc25C*, cyclin B1 and p21. Results indicated that CQ (> 0.25 mM) inhibited *cdc2*, *cdc25C* and cyclin B1 expression of pulp cells. However, CQ induced p21 mRNA expression of pulp cells (Fig 3A). We also evaluated the expression of related proteins by western blot analysis. We found that CQ inhibited *cdc2*, *cdc25C* and cyclin B1 protein expression of pulp cells, whereas CQ stimulated p21 protein expression (Fig 3B). Moreover, CQ also inhibited the expression of type I collagen, the important extracellular protein of pulp (Fig 3B). Immunofluorescent staining further showed increasing ATM phosphorylation (p-ATM) in the nucleus of pulp cells after exposure to CQ (Fig 3C). The ATM-phosphorylated p-Chk2 (red TRITC fluorescence) also increased in both nucleus and cytosol after exposure to 2 mM CQ (Fig 3C). The expression of p-p53 (red TRITC fluorescence) was slightly and gradually increased in the nucleus of pulp cells after incubation in 2 mM CQ (Fig 3C). A p53-regulated protein, GADD45 α (red TRITC fluorescence), was also boosted at 2 mM CQ. The expression of GADD45 α was extensive in the cytoplasm and nucleus (Fig 3C).

Effects of CQ on Oxidative Stress of Human Dental Pulp Cells

The fluorescence of DCF was expressed as the mean fluorescence of cells exposed to CQ relative to those in control group (control near 100). As shown in Fig 4A, exposure to 2 mM CQ elevated the intracellular ROS as revealed by the increase in DCF fluorescence. Quantitatively, exposure to higher concentrations of CQ for 3 hours led to a significant concentration-dependent increase of intracellular ROS. The mean DCF fluorescence increased markedly to about 128.02%, 148.38% and 164.12%, respectively, by the treatment of 0.5, 1 and 2 mM CQ ($p < 0.05$) for 3 hours (Fig 4B). Since 8-isoprostane has been shown to be an oxidative stress marker, we further studied and found the stimulation of 8-isoprostane production by CQ in dental pulp cells at concentrations of 1–3 mM (Fig 4C).

Effects of CQ on COX-2 Expression and PGE₂ Production of Pulp Cells

Since the exposure of dental pulp to composite resin or DBA may potentially induce pulpal inflammation, it is interesting to know whether CQ may induce COX-2 expression and PGE₂ production. Results showed that CQ stimulated COX-2 mRNA expression at concentrations higher than 1 mM (Fig 5A). Similarly, CQ also induced COX-2 protein expression at concentrations of 1–2 mM (Fig 5B). Accordingly, CQ also stimulated PGE₂ production of pulp cells at concentrations of 1 and 2 mM (Fig 5C) as well as PGF_{2 α} production (data not shown). However, inhibition of COX by aspirin was not able to prevent the CQ-induced cytotoxicity to pulp cells (Fig 5D).

Effects of NAC, Catalase and SOD on CQ-Induced Cytotoxicity of Pulp Cells

Cell viability was expressed as the percentage of control. After pre-treatment with 2.5 and 5 mM NAC for 30 minutes and then co-incubation with 2 mM CQ for 24 hours, the reduction of cell number caused by 2 mM CQ was significantly attenuated ($p < 0.05$) (Fig 6A). Besides, the decrease in viable cell number by CQ was also prevented by the pre-treatment and co-incubation with catalase (Fig 6B). SOD (500 U/ml) also decreased the CQ-induced cytotoxicity to pulp cells (Fig 6C). Moreover, NAC attenuated the CQ-induced p21 and HO-1 protein

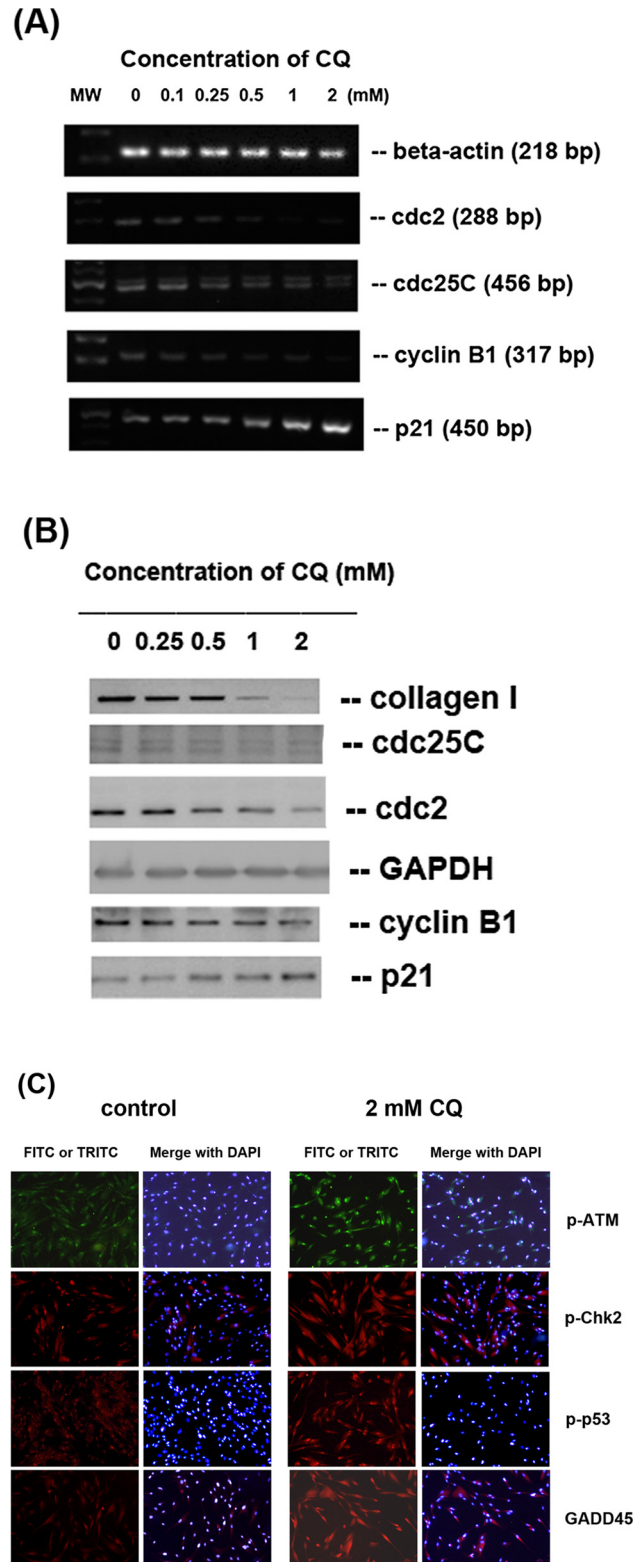


Fig 3. Effects of CQ on cell cycle-related genes and protein expression of human dental pulp cells. Human dental pulp cells were treated with CQ for 24 hours. **(A)** mRNA expression by RT-PCR analysis (cdc2, cdc25C, cyclin B1, p21 and beta-actin as control), **(B)** Western blotting analysis of protein expression (cdc2, cdc25C, cyclin B1, p21, collagen and GAPDH as control). **(C)** Immunofluorescent staining of p-ATM

(FITC, green), p-Chk2, p-p53 and GADD45 (TRITC, red) protein expression of pulp cells with/without exposure to 2 mM CQ for 24 hours. Cell nuclei were stained with DAPI (as blue fluorescence). One representative picture of RT-PCR, western blotting and IF was shown.

doi:10.1371/journal.pone.0143663.g003

expression, reversed the CQ-induced decline of collagen I expression, but showed little effect on CQ-induced decrease in cyclin B1, cdc2 and cdc25C expression of pulp cells ([Fig 6D](#)).

Stimulation of HO-1 by CQ in Dental Pulp Cells and Its Role in Toxic Effect

The expression of HO-1 was weak in control pulp cells. CQ (> 0.5 mM) markedly stimulated HO-1 mRNA expression of pulp cells as analyzed by RT-PCR ([Fig 7A](#)). Similarly CQ also induced HO-1 protein expression at concentrations higher than 0.5 mM, and even more obvious at 1–2 mM ([Fig 7B](#)). Moreover, the reduction of cell viability by 2 mM CQ was markedly enhanced after the pre-treatment/co-incubation with 5 μ M ZnPP (a HO-1 inhibitor) ([Fig 7C](#)). Accordingly, the CQ-induced PGE₂ production of pulp cells was also inhibited by ZnPP ([Fig 7D](#)), possibly due to cytotoxicity.

Role of MEK/ERK Signaling in Regulation of CQ-Induced Toxicity

In order to know the signal transduction pathways responsible for the CQ-induced cellular alteration, we first studied the MEK/ERK signaling, and found that CQ stimulated ERK1/ERK2 phosphorylation of pulp cells at concentrations higher than 0.25 mM ([Fig 8A](#)). U0126 (a MEK/ERK signaling inhibitor) was not able to prevent the CQ-induced cytotoxicity ([Fig 8B](#)). Intriguingly, U0126 could attenuate the CQ-induced COX-2 protein expression and PGE₂ production of pulp cells ([Fig 8C and 8D](#)).

Discussion

It has been found that photoinitiators and monomers may be released from dentin bonding agents and various resinous materials even after light curing. They may potentially affect the health of localized oral mucosa when released into saliva or the dental pulp tissues when the caries cavity is deep with thin remaining dentin thickness [24,25]. Destructive effects such as mineralization impairment, inflammation, abscess formation, and necrosis of the dental pulp are found when deep caries cavities are filled by DBA and resin composites [26–28]. The effect of photoinitiators and resin monomer on dental pulp cells may convey crucial clinical implications, but the underlying mechanisms await further investigations. In the present study we found one popularly-used photoinitiator, CQ, inducing marked cytotoxicity to human dental pulp cells at concentrations higher than 1 mM, and with morphological alterations, which are known to be crucial for cell proliferation, movement, as well as tissue morphogenesis [23]. These findings are in concordance with the morphological aberrations induced by hydroxyethylmethacrylate (HEMA), a common monomer in resin composites and dentin bonding agents (DBAs). Chang *et al.* presented apparent retraction and loss of the extended cellular process after treated with 10 mM HEMA for 24 hours [19]. Accordingly the proliferation of human dental pulp cells was obviously suppressed to about 70% and 50% of control after exposure to CQ (1–2 mM) for 24 hours, respectively. This observation is similar to the data reported on gingival fibroblasts [1,8]. Masuki *et al.* documented that the effective inhibitory concentration of CQ on human gingival fibroblasts was 1 mM [1], while Volk *et al.* reported that the ED₅₀ (effective dose, 50%) value of CQ to human gingival fibroblasts was about 2.7±0.8 mM [8].

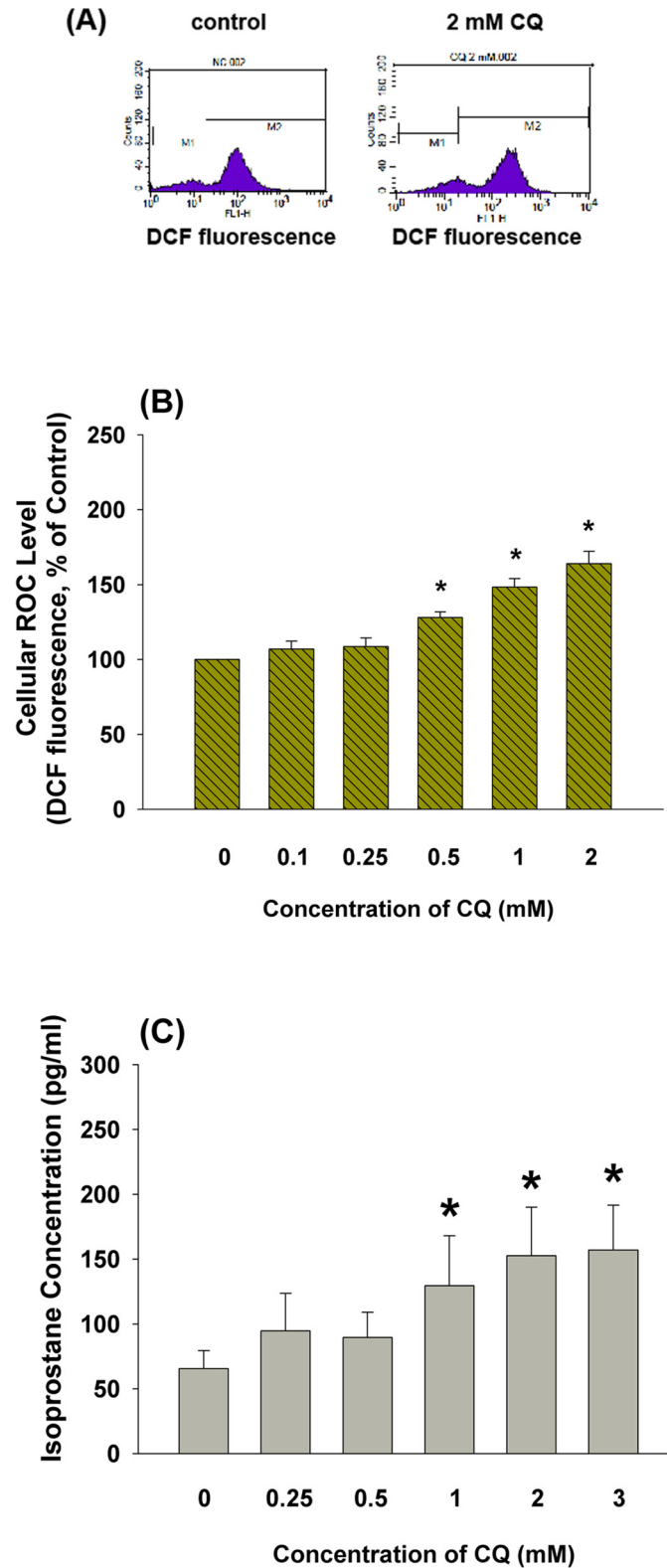


Fig 4. Effect of CQ on cellular oxidative stress of dental pulp cells. (A) One representative DCF flow cytometry profiles of pulp cells was shown. Human dental pulp cells were exposed to CQ for 3 hours. In each plot, there were 2 populations of cells, M1 and M2, with different intracellular DCF content. The mean fluorescence of cells in M2 population represented the DCF fluorescence of that group. **(B)** Quantitative

histogram of DCF assay ($n = 5$, Mean \pm SE, *denotes a statistically significant difference when compared with solvent-treated control ($p < 0.05$), (C) Stimulation of 8-isoprostane production of pulp cells by CQ. *denotes statistically significant difference when compared with control.

doi:10.1371/journal.pone.0143663.g004

The cytotoxicity by CQ can be partly explained by its induction of cell cycle arrest and apoptosis. Cell proliferation is tightly regulated by cell cycle progression and impairment of cell cycle control may result in growth arrest, cytotoxicity and even apoptosis [12]. Analysis of cell cycle alterations was therefore conducted to explore the cytotoxic mechanism of CQ. We interestingly found that CQ induced cell cycle arrest of human dental pulp cells. A G₀/G₁ cell cycle arrest was noted in cells treated with 0.5 mM CQ. In addition, higher concentrations of CQ (1 and 2 mM) induced G₂/M cell cycle arrest. Sub-G₀/G₁ population elevated in 1 and 2 mM CQ-treated groups, suggesting the presence of apoptotic cells. The induction of pulp cell apoptosis by CQ was further confirmed by the PI-Annexin V staining flow cytometry. Masuki *et al.* showed that 1 mM and 5 mM CQ induced a significant and sustained accumulation of human gingival fibroblasts in G₀/G₁ phase of the cell cycle after incubation for 24 hours [1]. The possible reasons for the difference may be due to different detoxifying enzymes in human dental pulp cells and human gingival fibroblasts. However, further studies are needed to make clear these points.

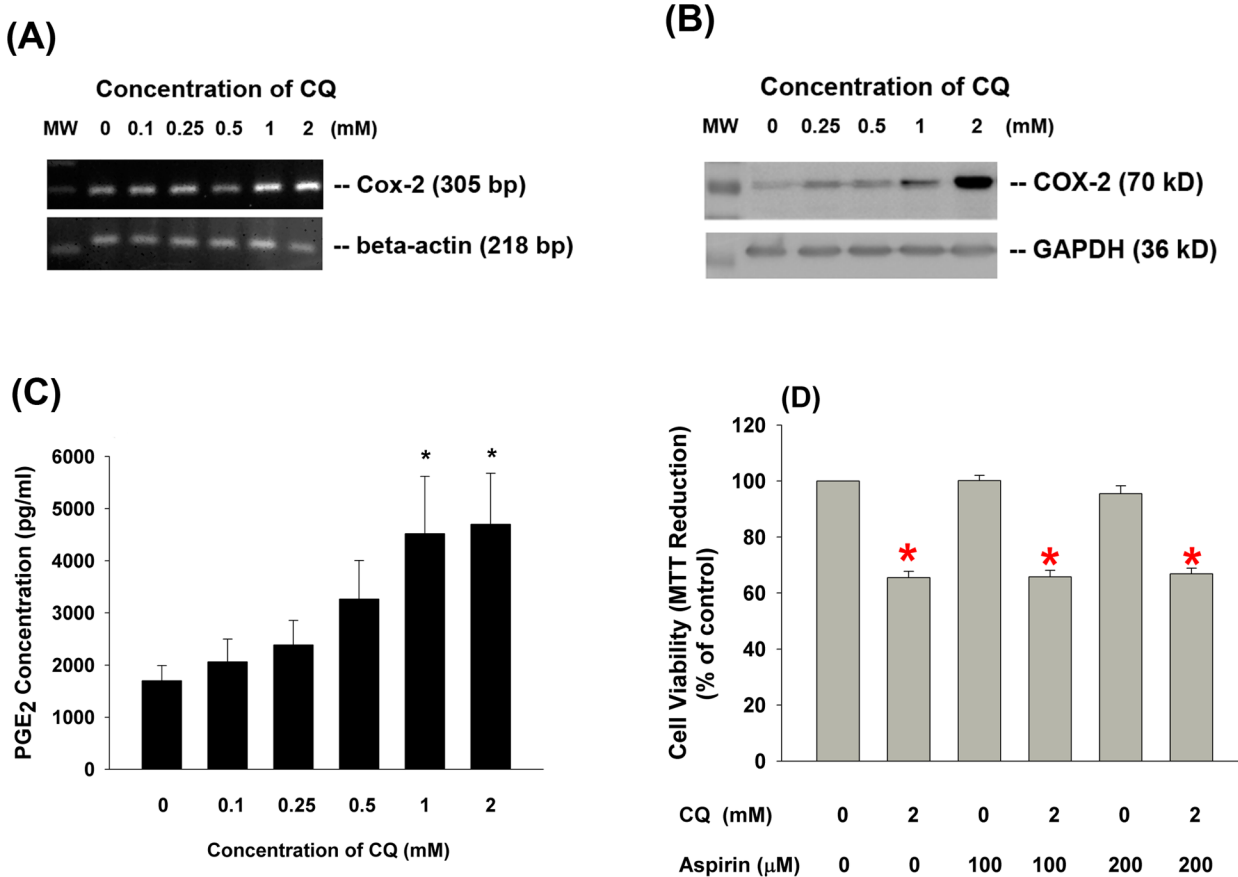


Fig 5. Effects of CQ on COX-2 expression and PGE₂ production of human dental pulp cells. Human dental pulp cells were treated with CQ for 24 hours. (A) COX-2 mRNA expression of pulp cells, (B) COX-2 protein expression of pulp cells, (C) PGE₂ production of pulp cells. (D) Effect of aspirin on CQ-induced cytotoxicity of pulp cells. Results were expressed as Mean \pm SE. *denotes statistically significant difference when compared with solvent control.

doi:10.1371/journal.pone.0143663.g005

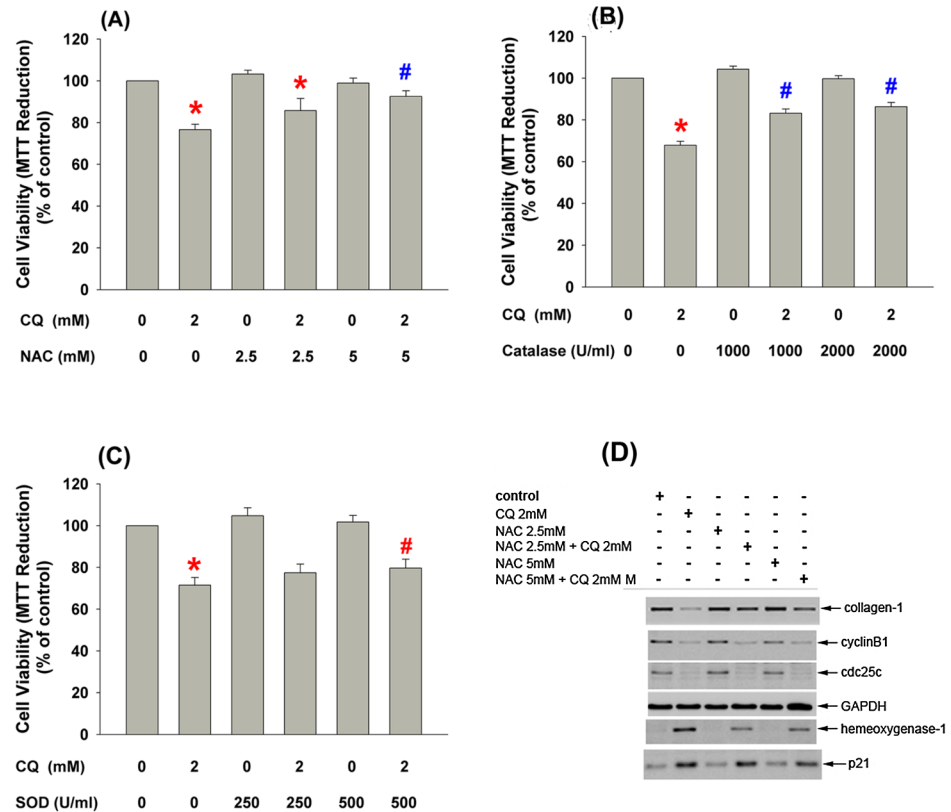


Fig 6. Effect of NAC, catalase and SOD on CQ-induced cytotoxicity of pulp cells (A) Effect of NAC on CQ-induced cytotoxicity of pulp cells, (B) Effect of catalase on CQ-induced cytotoxicity of pulp cells, (C) Effect of SOD on CQ-induced cytotoxicity of pulp cells. Results were expressed as cell viability (Mean \pm SE, % of control), *denotes a statistically significant difference when compared with solvent-treated control. #denotes a statistically significant difference when compared with CQ solely group ($p < 0.05$). (D) Effect of NAC on CQ-induced changes of p21, collagen I, cdc2, cdc25C and cyclin B1 protein expression. GAPDH expression was used as control. One representative western blotting result was shown.

doi:10.1371/journal.pone.0143663.g006

Little is known about the mechanisms responsible for CQ cytotoxicity. Cell cycle control and apoptosis are affected by the expression of various cyclins and cyclin-dependent kinases (Cdk) in specific cell cycle phases. A number of proteins are involved in cell cycle checkpoints to respond to DNA damage, recruit repair machinery, delay or arrest cell cycle progression, and even induce apoptosis [10]. For example, G₂/M transition is regulated by the mitosis promoting factor composed of cdc2 (CDK1) and cyclin B1. In this study, a marked dose-dependent inhibition of the expression of both cdc2 and cyclin B1 was observed after 24 hours exposure to CQ. In addition, CQ also inhibited the expression of type I collagen, a major extracellular protein of dental pulp, suggesting the effect of CQ on matrix turnover and pulpal repair. The inhibitory effect of CQ on cdc2 and cyclin B might therefore down-regulate the association of the mitosis promoting factor, and prevent the cell cycle progression, causing the human dental pulp cells to arrest in G₂/M phase. The mitosis promoting factor cdc2/cyclin B1 complex is kept inactive during G₂ phase by kinases Wee1 and Myt1 through phosphorylation on tyrosine 15 and threonine 14 of cdc2. At the onset of mitosis, the phosphatase cdc25C can activate cdc2/cyclin B complex via dephosphorylation of these residues on cdc2 [29]. Besides, it is known that cdc25C is the target of the acute and transient cell cycle delay for G₂/M phase.

In this study, CQ showed an inhibitory effect to cdc25C expression and stimulation of p21 that might inactivate cdc2/cyclin B1 complex. CQ might therefore induce G₂/M arrest and

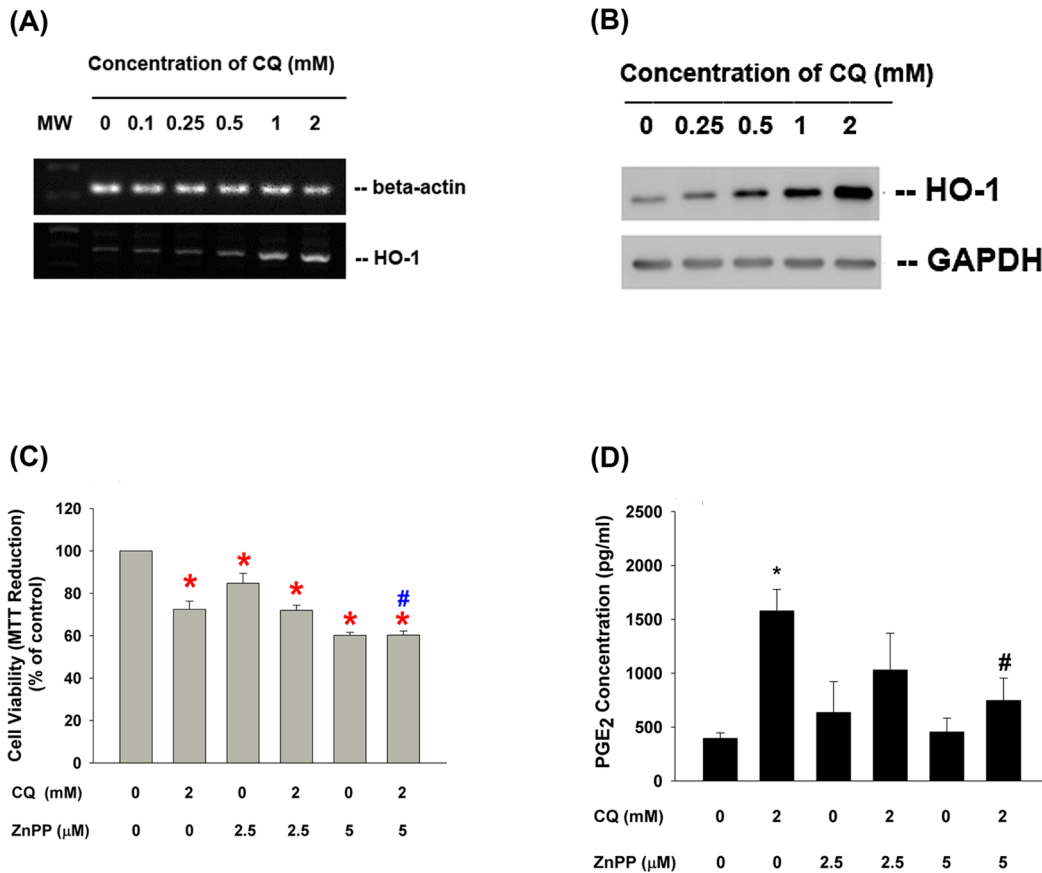


Fig 7. CQ induced HO-1 expression and its role in cytotoxicity, isoprostane, and PGE₂ production of pulp cells. (A) Stimulation of HO-1 mRNA expression by CQ in dental pulp cells. (B) Stimulation of HO-1 protein expression by CQ in dental pulp cells. (C) Effect of ZnPP on CQ-induced cytotoxicity, (D) Effect of ZnPP on CQ-induced PGE₂ production of pulp cells. *denotes the presence of statistically significant difference when compared with control. #denotes a statistically significant difference when compared with CQ solely group (p<0.05).

doi:10.1371/journal.pone.0143663.g007

apoptosis of pulp cells possibly via stimulation of p21 and inhibition of cdc2, cyclin B1 as well as cdc25C. In order to know more about the mechanisms of cell cycle arrest and apoptosis induced by CQ, we analyzed the changes in expression of upstream signaling molecules such as GADD, p53, Chk2 and ATM in dental pulp cells. As for the delayed and sustained cell cycle arrest for G₂/M phase, p53 can be phosphorylated and activated by Chk2 or directly by ATM. The transcriptional targets of p53 include p21, GADD45 as well as 14-3-3 σ , and they inactivate the cdc2/cyclin B complex through different ways [30]. The sustained pathway for prevention of cell mitosis is p53-dependent, in which the transcription of p21, GADD45 and 14-3-3 σ proteins is activated by p53, thereby inactivating or sequestering cyclin B/cdc2 complex [29]. In this study, GADD45 α protein expression was also stimulated by CQ. GADD45 bond both Cdks and proliferating cell nuclear antigen (PCNA), a protein involved in DNA replication and repair. GADD45 may inhibit cell cycle progression and promote DNA excision repair. This was possibly due to activation of upstream p53 molecule that may occur in response to DNA damage, hypoxia or other genotoxic stress. We further found the activation of ATM, Chk2 and p53 of pulp cells by CQ, indicating the triggering of DNA double strand breaks or other damages to pulp cells by CQ. Similarly Eckhardt et al. [31] have reported that TEGDMA may stimulate ROS production, induce DNA damage, and activate ATM, p38 and ERK in THP-1 mononuclear cells to control cell survival and apoptosis. These results suggest the

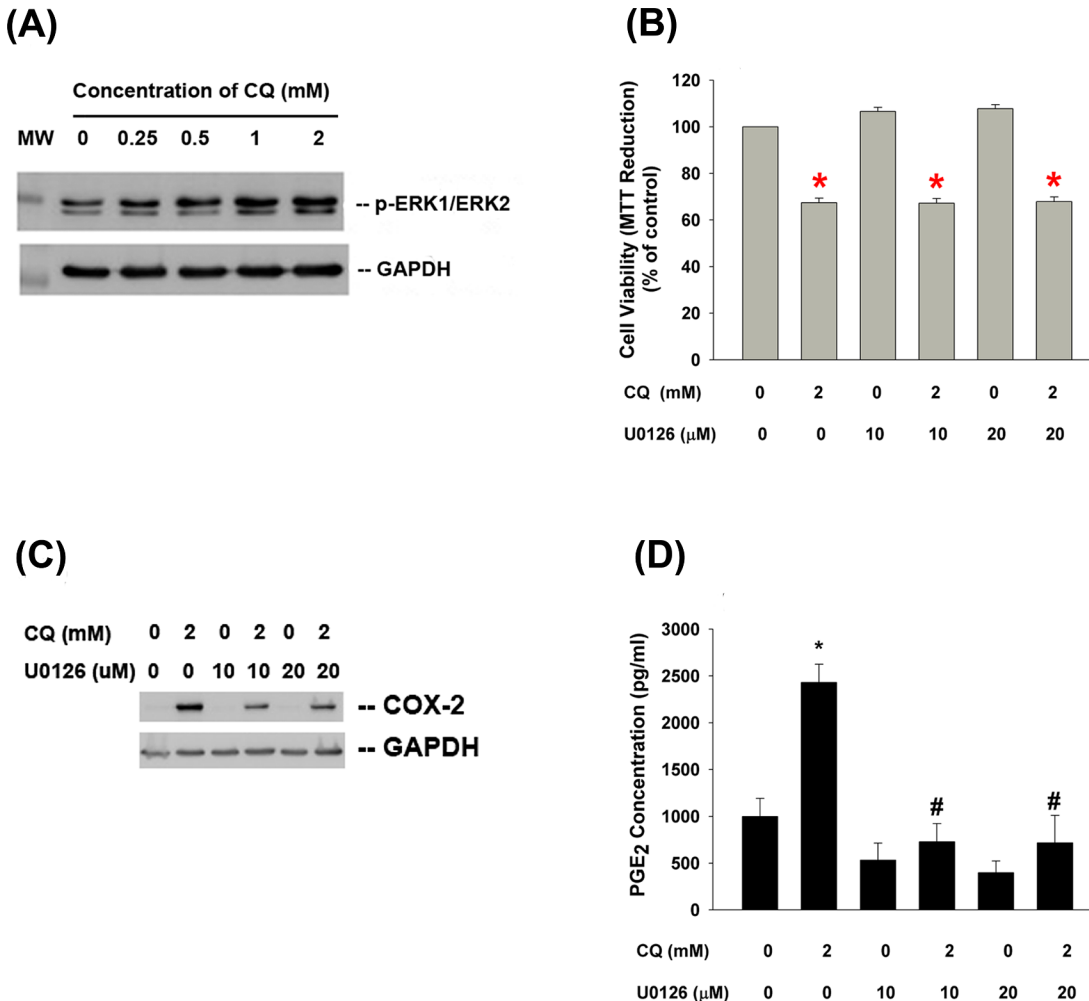


Fig 8. Role of MEK/ERK signaling on CQ-induced cytotoxicity, COX-2 expression and PGE₂ production of pulp cells. (A) Stimulation of ERK1/ERK2 phosphorylation by CQ, (B) Effect of U0126 on CQ-induced cytotoxicity of pulp cells, (C) Effect of U0126 on CQ-induced COX-2 protein expression of pulp cells. One representative western blotting result was shown. (D) Effect of U0126 on CQ-induced PGE₂ production of pulp cells. Results were expressed as Mean \pm SE, *denotes a statistically significant difference when compared with solvent-treated control. #denotes a statistically significant difference when compared with CQ solely group ($p < 0.05$).

doi:10.1371/journal.pone.0143663.g008

possible cytotoxic and genotoxic effect to dental pulp during clinical composite resin restoration procedures.

Since pulpal inflammation was often noticed when exposed pulp or deep caries cavity is restored with DBA and composite resins, we further studied and found that CQ stimulates prostaglandin E₂ (PGE₂) and PGF_{2 α} production of pulp cells. Prostaglandins (PGs) are important mediators to regulate pulp tissue inflammatory response [32], and prior study has found an increasing level of PGE₂ and PGF_{2 α} in the diseased processes of pulpitis [33]. DBA and resinous root canal sealers are also demonstrated to stimulate COX-2 expression in human osteoblasts [34]. Healthy dental pulp expresses little COX-2, whereas all inflamed dental pulps reveal elevated COX-2 expression [35]. As a major component of DBA and resin composites, CQ (>0.5 mM) is shown to induce COX-2 mRNA and protein expression in human dental pulp cells in this study. To the best of our knowledge, this is the first study to show stimulatory effect of CQ on COX-2 expression and prostanoids production. Both PGE₂ and PGF_{2 α} may involve in the pulpal inflammatory response possibly via activation of prostaglandin EP and FP

receptors, as well as stimulation of calcium and MEK/ERK signaling to affect pulpal functions, such as alkaline phosphatase activity and IL-8 production [14,16,21]. Addition of aspirin cannot prevent the CQ-induced cytotoxicity, further suggesting that COX activation is not involved in this process. These results suggest a potential role of CQ in inducing pulpal inflammation after composite resin restoration of dental caries.

Imbalance between ROS and antioxidants may lead to cellular oxidative stress that is crucial for induction of tissue inflammation, degenerative diseases, aging and carcinogenesis [36]. In this study, ROS production increased significantly after the exposure to 0.5–2 mM CQ for 3 hours as shown by elevation of cellular DCF fluorescence and 8-isoprostane production. This finding was generally in accordance to the published data [7,8]. Engelmann *et al.* reported that at concentrations higher than 1 mM, CQ caused a significant concentration-dependent increase of intracellular ROS after 1.5 hours [7]. On the other hand, Volk *et al.* found that at concentrations higher than 0.5 mM, CQ caused a significant concentration-dependent increase of intracellular ROS after 3 hours [8]. The mechanisms and the role of 8-isoprostane production in pulp cells and its role in regulation of pulpal physiology and pathological processes awaited further investigation. Since ROS production had been shown to mediate resin monomers-induced toxicity [25] and anti-oxidants had a potential to decrease their toxic effects [15,25], we further delineated the relationship between ROS formation and cytotoxicity caused by CQ. Interestingly, three ROS scavengers—NAC, catalase and SOD, all attenuated the CQ-induced cytotoxicity to dental pulp cells, suggesting the involvement of ROS in CQ cytotoxicity. Unexpectedly, NAC could attenuate the CQ-induced p21 and HO-1 protein expression, preventing the CQ-induced decline of collagen I expression, but showed little effect on CQ-induced decrease in cyclin B1, cdc2 and cdc25C expression of pulp cells. These results suggested that excessive amounts of ROS may lead to cell death via p21 and inhibit collagen formation that can be prevented by NAC/catalase/SOD. A small elevation of cellular ROS was enough to inhibit cyclin B1, cdc2, cdc25C expression, whereas excessive amounts of ROS were needed to stimulate p21, HO-1 and inhibit collagen I that can be more easily prevented by NAC.

HO-1 is known to be an inducible isoform of heme oxygenase in response to oxidative stress and is a protective enzyme against oxidant-induced injury [9]. HO-1 may catalyze the decomposition of heme to generate iron, carbon monoxide and biliverdin, which have the activity to promote cell viability, circulatory integrity and inhibit inflammation [37]. Intriguingly, we found that CQ (> 0.5 mM) stimulated HO-1 mRNA and protein expression, suggesting an adaptive response of dental pulp to CQ. Furthermore, we analyzed the role of HO-1 expression on CQ-induced cytotoxicity by pretreatment and co-incubation with/without ZnPP, a HO-1 inhibitor. We found that the reduction of cell proliferation caused by 2 mM CQ was markedly enhanced by 5 μ M ZnPP, indicating the increase of HO-1 expression might play a protective role against CQ-induced cytotoxicity. This may also partly explain the inhibition of CQ-induced PGE₂ production of pulp cells by ZnPP.

Recently resin monomer—HEMA has been shown to induce ERK activation, but to inhibit protein kinase B (Akt) activity of pulp cells, and both of them may affect the cytotoxicity of HEMA [38]. Suppression of ERK and phosphatidylinositol 3 kinase/Akt signaling pathways also exerts differential effects on the triethylene glycol dimethacrylate (TEGDMA)-induced cytotoxicity to pulp cells [39]. Stimulation of ROS production by HEMA and TEGDMA further activates ERK1/2, JNK and p38 to regulate the cytotoxicity and apoptosis of salivary gland cells [40]. All the above suggest the involvement of mitogen-activated protein kinases (MAPKs) and PI3K/Akt signaling pathways in the resin monomers- and DBAs-induced cell alterations. We further tested whether CQ-induced COX-2 expression and PGE₂ production was mediated by MEK/ERK1/2 signaling. Intriguingly CQ induced ERK phosphorylation of

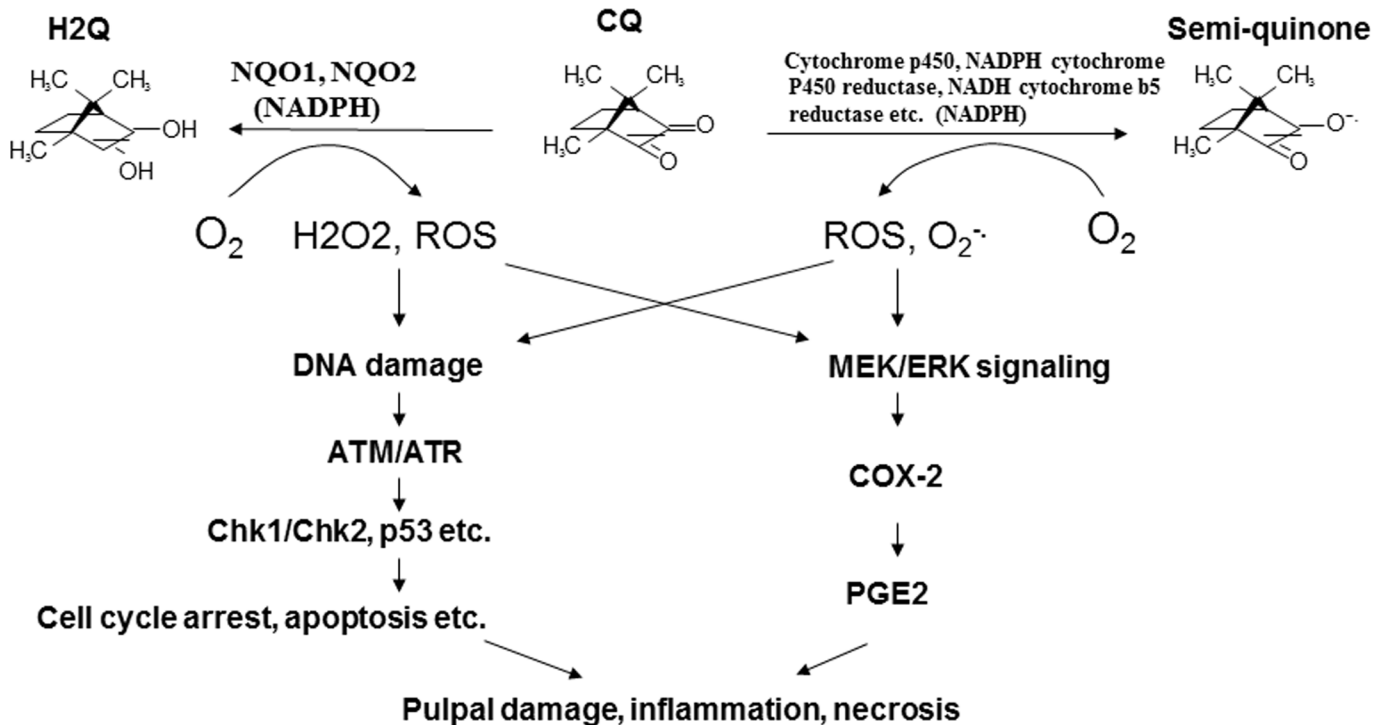


Fig 9. Mechanisms of CQ-induced cytotoxicity and prostaglandins production in dental pulp cells. CQ can be metabolized by one electron reduction enzymes (NQO1, NQO2) or 2 electron reduction enzymes (cytochrome P450, NADPH cytochrome P450 reductase etc.) to generate hydroquinone and semiquinone radicals with concomitantly production of ROS (H₂O₂, superoxide etc.). These ROS may involve in cytotoxicity and tissue inflammation via stimulation of DNA damage, ATM/Chk2/p53 and MEK/ERK signaling.

doi:10.1371/journal.pone.0143663.g009

pulp cells and pretreatment/co-incubation with U0126, a MEK/ERK signaling inhibitor, and markedly suppressed the CQ-induced COX-2 expression and PGE₂ production. However, U0126 showed little preventive effect on CQ-induced cytotoxicity. This suggested that CQ-induced COX-2 expression and PGE₂ production of pulp cells were mediated by MEK/ERK signaling. CQ induced ROS generation to mediate cytotoxicity by pathways other than MEK/ERK.

Conclusions

In conclusion, CQ may cause cytotoxicity, cell cycle arrest, apoptosis and PGE₂ production of pulp cells. These can be due to stimulation of ROS and isoprostane production, activation of ATM/Chk2 signaling, induction of HO-1, COX-2 and p21 expression, and the inhibition of cdc2, cdc25C, cyclin B1 (Fig 9). These results are important for our understanding of the mechanism of pulp necrosis and tissue inflammation after clinical operative restoration of dental caries by dentin bonding agent and composite resin.

Acknowledgments

This study is supported by grants from Chang Gung Memorial Hospital (CMRPF1C0011, CMRPF1C0012, NMRPF3C0091, NMRPF3C0061) and Ministry of Science and Technology (NSC102-2314-B-255-003-MY2, NSC102-2628-B-255-001-MY3, NSC101-2320-B-255-002, NSC-100-2314-B-002-094, NSC-101-2320-B-255-002, and NSC102-2314-B002-051-MY2), National Taiwan University Hospital (NTUH) and National Taiwan University Hospital (NTUH).

Author Contributions

Conceived and designed the experiments: MCC JHJ. Performed the experiments: MCC LDL CPC MTW HHC MSL TYS PYJ SYY. Analyzed the data: MCC LDL JHJ PYJ SYY HJL. Contributed reagents/materials/analysis tools: MCC JHJ CPC LDL MTW HHC MSL TYS PYJ SYY. Wrote the paper: MCC JHJ.

References

- Masaki K, Nomura Y, Bhawal UK, Sawajiri M, Hirata I, Nahara Y, Okazaki M (2007). Apoptotic and necrotic influence of dental resin polymerization initiators in human gingival fibroblast cultures. *Dent Mater J* 26: 861–869. PMID: [18203492](#)
- Taira M, Urabe H, Hirose T, Wakasa K, Yamaki M (1988). Analysis of photo-initiators in visible-light-cured dental composite resins. *J Dent Res* 67: 24–28. PMID: [11039039](#)
- Stansbury JW (2000). Curing dental resins and composites by photopolymerization. *J Esthetic Dent* 12: 300–308.
- Spahl W, Budzikiewicz H, Geurtsen W (1998). Determination of leachable components from four commercial dental composites by gas and liquid chromatography/mass spectrometry. *J Dent* 26: 137–145. PMID: [9540311](#)
- Michelsen VB, Moe G, Skålevik R, Jensen E, Lygre H (2007). Quantification of organic elutes from polymerized resin-based dental restorative materials by use of GC/MS. *J. Chromatogr. (B)* 850: 83–91.
- Geurtsen W, Lehmann F, Spahl W, Leyhausen G (1998). Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. *J Biomed Mater Res* 41: 474–480. PMID: [9659618](#)
- Engelmann J, Volk J, Leyhausen G, Geurtsen W (2005) ROS formation and glutathione levels in human oral fibroblasts exposed to TEGDMA and camphorquinone. *J Biomed Mater Res (B): Appl Biomater* 75B: 272–276.
- Volk J, Ziemann C, Leyhausen G, Geurtsen W (2009). Non-irradiated camphorquinone induces DNA damage in human gingival fibroblasts. *Dent Mater* 25: 1556–1563. doi: [10.1016/j.dental.2009.07.009](#) PMID: [19716169](#)
- Chung SW, Hall SR, Perrella MA (2009). Role of haemoxygenase-1 in microbial host defense. *Cell Microbiol* 11: 199–207. doi: [10.1111/j.1462-5822.2008.01261.x](#) PMID: [19016784](#)
- Vermeulen K, Bockstaele DRV, Berneman ZN (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolifer* 36: 131–149.
- Nyberg KA, Michelson RJ, Putnam CW, Weinert TA (2002). Toward maintaining the genome: DNA damage and replication checkpoints. *Ann Rev Genet* 36: 617–656. PMID: [12429704](#)
- Lukas J, Lukas C, Bartek J (2004) Mammalian cell cycle checkpoints: signaling pathways and their organization in space and time. *DNA Repair* 3: 997–1007. PMID: [15279786](#)
- Chang MC, Lin LD, Chan CP, Chang HH, Chen LI, Lin JH, Yeh HW, Tseng WY, Lin PS, Lin CC, Jeng JH (2009). The effect of BisGMA on cyclooxygenase-2 expression, PGE2 production and cytotoxicity via reactive oxygen species- and MEK/ERK-dependent and-independent pathways. *Biomaterials* 30: 4070–4077. doi: [10.1016/j.biomaterials.2009.04.034](#) PMID: [19467701](#)
- Chang MC, Chang HH, Lee MY, Lin CC, Yeh HW, Yang TT, Lin PS, Tseng WY, Jeng JH (2009). Prostaglandin F(2alpha)-induced interleukin-8 production in human dental pulp cells is associated with MEK/ERK signaling. *J Endod* 35: 508–512. doi: [10.1016/j.joen.2008.12.023](#) PMID: [19345795](#)
- Chang MC, Chen LI, Chan CP, Lee JJ, Wang TM, Yang TT, Lin PS, Lin HJ, Chang HH, Jeng JH (2010). 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* 31: 8164–8171. doi: [10.1016/j.biomaterials.2010.07.049](#) PMID: [20673999](#)
- Chang MC, Chen YJ, Lee MY, Lin LD, Wang TM, Chan CP, Tsai YL, Wang CY, Lin BR, Jeng JH (2010). Prostaglandin D(2alpha) stimulates MEK-ERK signaling but decreases the expression of alkaline phosphatase in dental pulp cells. *Int Endod J* 43: 461–468. doi: [10.1111/j.1365-2591.2010.01699.x](#) PMID: [20536573](#)
- Chang MC, Lin LD, Chuang FH, Chan CP, Wang TM, Lee JJ, Jeng PY, Tseng WY, Lin HJ, Jeng JH (2012). Carboxylesterase expression in human dental pulp cells: Role in regulation of BisGMA-induced prostanoid production and cytotoxicity. *Acta Biomater* 8: 1380–1387. doi: [10.1016/j.actbio.2011.09.011](#) PMID: [21951919](#)
- Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55–63. PMID: [6606682](#)

19. Chang HH, Guo MK, Kasten FH, Chang MC, Huang GF, Wang YL, Wang RS, Jeng JH (2005) Stimulation of glutathione depletion, ROS production and cell cycle arrest of dental pulp cells and gingival epithelial cells by HEMA. *Biomaterials* 26: 745–753. PMID: [15350779](#)
20. Chang HH, Chang MC, Wang HH, Huang GF, Lee YL, Wang YL, Chan CP, Yeung SY, Tseng SK, Jeng JH (2014), Urethane dimethacrylate induces cytotoxicity and regulates cyclooxygenase-2, hemeoxygenase and carboxylesterase expression in human dental pulp cells. *Acta Biomater* 10: 722–731. doi: [10.1016/j.actbio.2013.10.006](#) PMID: [24140606](#)
21. Chang MC, Chen YJ, Tai TF, Tai MR, Li MY, Tsai YL, Lan WH, Wang YL, Jeng JH (2006). Cytokine-induced prostaglandin E2 production and cyclooxygenase-2 expression in dental pulp cells: downstream calcium signaling via activation of prostaglandin EP receptor. *Int Endod J* 39: 819–826. PMID: [16948668](#)
22. McGarvey TW, Malkowicz SB (2005). Steady state expression of cell-cycle regulatory genes in prostate carcinoma cell lines. *Urol Oncol* 5: 71–77.
23. McClay DR (1999). The role of thin filopodia in motility and morphogenesis. *Exp Cell Res* 253: 296–301. PMID: [10585250](#)
24. Geurtsen W, Spahl W, Muller K, Leyhausen G (1999). Aqueous extracts from dentin adhesives contain cytotoxic chemicals. *J Biomed Mater Res* 48: 772–777. PMID: [10556839](#)
25. Krifka S, Spagnuolo G, Schmalz G, Schweikl H (2013). A review of adaptive mechanisms in cell responses towards oxidative stress caused by dental resin monomers. *Biomaterials* 34: 4555–4563. doi: [10.1016/j.biomaterials.2013.03.019](#) PMID: [23541107](#)
26. Ferracane JL (2006). Hygroscopic and hydrolytic effects in dental polymer networks. *Dent Mater* 22: 211–222. PMID: [16087225](#)
27. Hebling J, Giro EM, Costa CA (1999). Biocompatibility of an adhesive system applied to exposed human dental pulp. *J Endod* 25: 676–682. PMID: [10687527](#)
28. Silva GA, Lanza LD, Lopes-Junior N, Moreira A, Alves JA (2006). Direct pulp capping with a dentin bonding system in human teeth: a clinical and histological evaluation. *Oper Dent* 31: 297–307. PMID: [16802637](#)
29. Taylor WR, Stark GR (2001). Regulation of the G₂/M transition by p53. *Oncogene* 20: 1803–1815. PMID: [11313928](#)
30. Cheng M, Olivier P, Diehl JA, Fero M, Roussel MF, Roberts JM, et al. (1999) The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J* 18: 1571–1583. PMID: [10075928](#)
31. Eckhardt A, Gerstmayr N, Hiller K, Bolay C, Waha C, Spagnuolo G, et al. (2009) TEGDMA-induced oxidative DNA damage and activation of ATM and MAP kinases. *Biomaterials* 30: 2004–2016.
32. Ricciotti E, FitzGerald GA (2011). Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol* 31: 986–1000. doi: [10.1161/ATVBAHA.110.207449](#) PMID: [21508345](#)
33. Miyauchi M, Takata T, Ito H, Ogawa I, Kobayashi J, Nikai H, et al. (1996) Immunohistochemical demonstration of prostaglandins E2, F2 alpha, and 6-keto-prostaglandin F1 alpha in rat dental pulp with experimentally induced inflammation. *J Endod* 22: 600–602. PMID: [9198415](#)
34. Huang FM, Chou MY, Chang YC (2003). Induction of cyclooxygenase-2 mRNA and protein expression by epoxy resin and zinc oxide-eugenol based root canal sealers in human osteoblastic cells. *Biomaterials* 24: 1869–1875. PMID: [12615477](#)
35. Guven G, Altun C, Gunhan O, Gurbuz T, Basak F, Akbulut E, et al. (2007) Co-expression of cyclooxygenase-2 and vascular endothelial growth factor in inflamed human pulp: an immunohistochemical study. *J Endod* 33: 18–20. PMID: [17185121](#)
36. Valko M, Leibfriz D, Moncol J, Cronin MT, Mazur M, Telser J (2007). Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39: 44–84. PMID: [16978905](#)
37. Ferenbach DA, Kluth DC, Hughes J (2010). Hemeoxygenase-1 and renal ischaemia-reperfusion injury. *Nephron Exp Nephrol* 115: e33–e37. doi: [10.1159/000313828](#) PMID: [20424481](#)
38. Spagnuolo G, D'Anto V, Valletta R, Strisciuglio C, Schmalz G, Schweikl H, et al. (2008) Effect of 2-hydroxyethyl methacrylate on human pulp cell survival pathways ERK and AKT. *J Endod* 34: 684–688. doi: [10.1016/j.joen.2008.02.040](#) PMID: [18498889](#)
39. Spagnuolo G, Galler K, Schmalz G, Cosentino C, Rengo S, Schweikl H (2004). Inhibition of phosphatidylinositol 3-kinase amplifies TEGDMA-induced apoptosis in primary human pulp cells. *J Dent Res* 83: 703–707. PMID: [15329376](#)
40. Samuelsen JT, Dahl JE, Karlsson S, Morisbak E, Becher R (2007). Apoptosis induced by the monomers HEMA and TEGDMA involves formation of ROS and differential activation of the MAP-kinases p38, JNK and ERK. *Dent Mater* 23: 34–39. PMID: [16430953](#)