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Original Article

Camphorquinone-induced phospholipase A_2 and cyclooxygenase −2 expression and its roles in 8-isoprostane and prostaglandin E_2 production of human dental pulp cells

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Abstract *Background/purpose:* Camphorquinone (CQ) is a widely-used photo-initiator in dentin bonding agent (DBA) and composite resin to promote resin polymerization and restore tooth decay. CQ may potentially induce pulpal inflammation and affect viability of dental pulp especially when the remaining dentin is minimal. Phospholipase A2 (PLA2) and cyclooxygenase-2 (COX-2) are crucial enzymes for tissue inflammation by inducing prostaglandins' production. However, little is known about the PLA2 isoforms' expression and their response to CQ in the dental pulp.

Materials and methods: Human dental pulp cells (HDPCs) were exposed to various concentrations of CQ with/without inhibitors (aspirin, eugenol or ASB14780) for 24 h. Protein and mRNA expression of cyclooxygenase-2 (COX-2), cPLA2, sPLA2 and iPLA2 were investigated by immunofluorescent staining and real-time PCR, respectively. Culture medium was collected for analysis of 8-isoprostane and PGE_2 production by enzyme-linked immunosorbant assay. Cell layer was utilized for MTT assay of cell viability.

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Results: CQ regulated the mRNA and protein expression of COX-2, cPLA2, sPLA2 and iPLA2 at concentrations of 2 and 3 mM. Aspirin, eugenol and ASB14780 (a PLA2 inhibitor) differentially attenuated the CQ-induced PGE_2 production in HDPCs. Aspirin, but not ASB14780 and eugenol, inhibited CQ-induced 8-isoprostane production. On the other hand, eugenol, ASB14780, and aspirin showed little effect on CQ-induced cytotoxicity to HDPCs.
Conclusion: CQ may possibly stimulate pulpal inflammation and necrosis by induction of cytotoxicity, 8-isoprostane and PGE2 production that are differentially regulated by various PLA2s. Aspirin, eugenol and PLA2 inhibitors may have potential therapeutic use to control pulpal inflammation after composite resin restoration.

Introduction

Composite resin and dentin bonding agents (DBAs) are currently the most popularly-used materials for restoration of tooth decay due to dental caries, tooth fracture and cervical abrasion. Photo-initiators such as camphorquinone (CQ) are often added into composite resin and DBAs for inducing resin polymerization by light curing. The polymerization of DBAs and composite resin is not complete and the contents of DBAs and composite resins such as resin monomers and CQ may be released into dental pulp or saliva even after polymerization. The released resin components may potentially affect the biological activity of oral mucosa, as well as the repair, inflammation, vitality of the dental pulp, especially when the reminaing dentin is minimal.[1,2] However, clinically it is very difficult to accurately determine the remaining dentin thickness between tooth decay and the dental pulp.

CQ, chemical structure as 1,7,7-trimethyl- bicyclo-[2,2,1]-hepta-2,3-dione, is a major component and photo-initiator in composite resin and DBAs. CQ in the composite resin is reported ranging from 0.17 % to 1.03 % w/w.[3] CQ concentration in eluates of composite resin was analyzed by gas-chromatography mass-spectroscopy and reported to be about 0.06–0.1 % (v/v), equivalent to 3–5 mM.[3] Since CQ concentrations in most light-curing composite resin are about 0.2–1.0 %, the calculated maximal concentration of CQ leaching from the composite resin was reported to be about 14 mM.[4,5] CQ, at relevant concentrations, may pose potential cytotoxicity and genotoxicity to the oral mucosa cells or human dental pulp cells.[5–7] Accordingly CQ was found to induce cytotoxicity, cell cycle aberration, autophagy, as well as 8-isoprostane, prostaglandin E2 (PGE2) and cathepsin L production in human dental pulp cells (HDPCs). These events are associated with reactive oxygen species (ROS) production, ATM/Chk2, MEK/ERK and hemeoxygenase-1 (HO-1) expression,[7,8] and contribute to pulpal inflammation and necrosis.

The 8-Isoprostane, as prostaglandin-like compound and oxidative stress biomarker, is produced generally through free radical-catalyzed peroxidation of free fatty acid such as arachidonic acid (AA). 8-isoprostance is reported to exhibit inflammatory effect and mediate airway smooth muscle contraction possibly via thromboxane A2 receptors.[9] Its roles in the dental pulp await further investigation. On the other hand, PGE2 may induce vasodilatation, increases vascular permeability, bone resorption, chemotaxis of inflammatory cells, and pain in affected tissues including the dental pulp.[10] The rate-limiting step in the formation of PGs (PGE2 and $PGF_{2\alpha}$) from AA is catalyzed by phospholipase A2 (PLA2) and cyclooxygenase (COX). Previous studies have found the stimulation of COX-2 expression of HDPCs by CQ and resin monomers.[7,11] There are more than 50 enzymes in PLA2 family, where cytosolic PLA2 (cPLA2), secretory PLA2 (sPLA2) and calcium-independent PLA2 (iPLA2) are three major PLA2.[12] These PLA2 family members show differential tissue distribution and play critical roles in the generation of lipid inflammatory mediators, and regulate membrane remodeling, bioenergetics, body surface barrier, as well as various cellular biological activities.[12] However, limited studies have evaluated expression various PLA2 in the dental pulp and their roles in pulpal physiology and pathology.

Arachidonic acid (AA) is released from cell membrane and organelles' membrane by various PLA2. AA can be further metabolized by COX-2 or free radical-mediated lipid peroxidation to generate various prostaglandins and 8-isoprostane,[7,11,12] two inflammatory mediators in tissue and pulpal inflammation." We hypothesize that CQ may induce pulpal inflammation and even necrosis of the dental pulp by its induction of 8-Isoprostane and PGE2 production as well as cytotoxicity. These events are possibly associated with differential PLAs and COX-2 induction. Pharmacological inhibition of PLA2 and COX-2 can be potentially used to prevent pulpal inflammatory response and even necrosis after operative restoration.

Materials and methods

Materials

Aspirin, CQ, eugenol, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl -tetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA, USA). Superscript TM III First Strand Synthesis System was bought from Invitrogen (Thermo Fisher Scientific Inc.). SYBR green real-time PCR kits were purchased

from PCR Biosystems Inc. (Wayne, PA, USA). RNA isolation kit and NucleoSpin RNA II were from Macherey—Nagel Inc. (Easton, PA, USA). ASB14780, and PGE2 and 8-isoprostane ELISA kits were bought from Cayman Chemical Company (Ann Arbor, MI, USA). Antibodies of cPLA2, sPLA2 and iPLA2 were purchased from Abclonal Science Inc. (Woburn, MA, USA) and GAPDH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CQ was dissolved in DMSO as stock solution and kept in brown bottle at −20 °C freezer.

Culture of human dental pulp cells

After the approval of Ethics Committee, National Taiwan University Hospital, human teeth were got from either extracted third molars or premolars of patients with signed informed consent. We used a hammer to split the teeth and get the dental pulp tissues. Pulp tissues were cut to small pieces by surgical knife and digested by culture medium containing 50 mg/ml type IV collagenase in an incubator for 1 h. After centrifugation, tissues were washed and resuspended in DMEM containing 10 % FBS and 1x penicillin/streptomycin and cultured in incubator. Culture medium was changed every 3—4 days. When the outgrowth of human dental pulp cells (HDPCs) in the culture dishes reached near confluence, they were detached by trypsin/EDTA treatment for frozen or sub-cultured for experiments. HDPCs at passages from 3rd to 8th were used in our experiments.[7,8]

Effect of camphorquinone on cyclooxygenase-2 and different phospholipase A2 mRNA expression in human dental pulp cells

To clarify further about whether CQ may influence COX-2 and various PLA2s expression, real-time PCR analysis was conducted. Briefly, HDPCs were treated as before and total RNA was isolated using Qiagen RNA isolation kit. After reverse transcription of RNA with Invitrogen SuperscriptTM III First Strand Synthesis System, the generated cDNA was amplified by real-Time PCR using SYBR green real-time PCR kit in a reaction mixture comprising SYBR master mix, specific primers, cDNA and DEPC water. The PCR reaction was set at stage 1: 95 °C for 2 min, 1 cycle; then stage 2: 95 °C 5 s, 60 °C for 30 s for 40 cycles.[13,14] The sequence of forward and reverse Primers for cPLA2: CCAAAGTGACAAAGGGGGCC and GCTACCACAGGC ACATCACG; For sPLA2: ATGAAGACCCTCCTACTG and TCAGCAACGAGGGGTGCT.[15] iPLA2-A: TTTGGCCG CCTGGTCAATAC and CTCCCGAACTCGGTCACTC,[16] COX-2 and β-actin (BAC, internal control).[14] For quantification, the Delta/Delta Cyclic threshold values (ΔΔCt = mean ΔCt [treated] - mean delta (Δ) Ct [control]) was used to determine the change in the level of gene expression. The fold changes of the experimental groups relative to the control (solvent) group were calculated using $2^{-\Delta\Delta Ct}$ method. The BAC gene was used as an internal control gene in all PCR experiments.

Effect of camphorquinone on different phospholipase A2 protein expression in human dental pulp cells

In short, HDPCs (1×10^5 cells/well) were seeded into 24-well culture plate with coverslips. Twenty-four hours later, cells were changed with fresh medium containing solvent (dimethyl-sulfoxide [DMSO]) control or CQ (0.5, 1, and 2 mM) for 24 h. The protein expression of three different PLA2 (cPLA2, sPLA2 and iPLA2) were investigated by immunofluorescent staining as described before,[14,17] using rabbit anti-human cPLA2, sPLA2 and iPLA2 (1:500, v/v) antibodies for overnight. After washing, HDPCs were stained with Fluorescein (FITC) AffiniPure™ Goat Anti-Rabbit IgG (H + L). The pictures were photographed under an immuno-fluorescent microscope.

Effect of aspirin, eugenol and ASB14780 on camphorquinone-induced cytotoxicity to human dental pulp cells

HPDCs (2.5×10^5 cells/well) were seeded into 6-well culture plate. After 24-hr, culture medium was changed (respectively) and then solvent control, aspirin, eugenol or ASB14780 were added and incubated for 30 min. Then CQ (2 mM) or solvent control were added and further co-incubated for 24 h. Cell viability of HDPCs was determined by MTT assay as described previously.[13,18] Results of cell viability were expressed as percentage of control (as 100 %). The results can be useful to know whether PLA2 may prevent or enhance the cytotoxicity of CQ.

Effect of aspirin, eugenol and ASB14780 on camphorquinone-induced 8-isoprostane and prostaglandin E2 production of human dental pulp cells

HDPCs (2.5×10^5 cells/6-well, 2 ml medium) were treated with solvent control, aspirin, eugenol or ASB14780 (a PLA2 inhibitor) for 30 min, and then treated with or without CQ (2 mM) or solvent control for further 24 h. Culture medium was collected for measurement of 8-isoprostane and PGE2 production of HDPCs by ELISA.[18]

Statistical analysis

Three or more independent experiments were performed for different assays. Results are expressed as Mean ± SE and analyzed by One-way ANOVA and post-hoc Tukey test. A P value < 0.05 is considered to have statistically significant difference between two comparing groups.

Results

Effect of camphorquinone on cyclooxygenase-2, and different phospholipase A2 mRNA expression of human dental pulp cells

Previous study has reported the stimulation of COX-2 mRNA expression in HDPCs by reverse transcription-PCR.[7] Accordingly we found the stimulation of COX-2 mRNA expression by real-time PCR. The maximal stimulation of COX-2 mRNA was found at a CQ concentration of 2 mM (Fig. 1A). CQ also induced the cPLA2 mRNA expression by 2.4-fold at a concentration of 2 mM ($P > 0.05$) (Fig. 1B). Moreover, CQ significantly stimulated the sPLA2 and iPLA2

mRNA expression of HDPCs at concentrations of 2 and 3 mM ($P < 0.05$) (Fig. 1C–and D).

Effect of camphorquinone on different phospholipase A2 protein expression of human dental pulp cells

CQ provoked the cPLA2 protein expression of HDPCs at concentrations of 1 mM and 2 mM, as indicated by increase in cellular green fluorescence in the cytosol and evident perinuclear localization (Fig. 2A). CQ also stimulated sPLA2 at concentrations of 1 and 2 mM as indicated by increase in green fluorescence (Fig. 2B), with a decrease in cell density. Accordingly, CQ further induced iPLA2 protein expression of HDPCs at concentrations of 1 and 2 mM (Fig. 2C).

Effect of aspirin, eugenol and ASB14780 on camphorquinone-induced 8-isoprostane production of human dental pulp cells

CQ was found to stimulated 8-isoprostane production of HDPCs.[7] We intriguingly found that CQ-induced 8-isoprostane production was effectively inhibited by aspirin (Fig. 3A). Unexpectedly eugenol showed no inhibitory effect on CQ-induced 8-isoprostane production in HDPCs (Fig. 3B). ASB14780 (5 and 10 μM) mildly attenuated the CQ-induced 8-isoprostane production in HDPCs with 19-25 % of inhibition ($P > 0.05$) (Fig. 3C).

Effect of aspirin, eugenol and ASB14780 on camphorquinone-induced prostaglandin E2 production of human dental pulp cells

CQ has been shown to stimulate PGE2 production of HDPCs.[7] In this study, we further found that CQ-induced PGE2 production was almost completely inhibited by aspirin (100 and 200 μM), a COX inhibitor with 90-100 % of inhibition ($P > 0.05$) (Fig. 4A). Eugenol also markedly suppressed the CQ-induced PGE2 production in HDPCs ($P < 0.05$) (Fig. 4B). On the other hand, ASB14780 (5 and 10 μM) mildly inhibited the CQ-induced PGE2 production in HDPCs with 22-29 % inhibition ($P > 0.05$) (Fig. 4C).

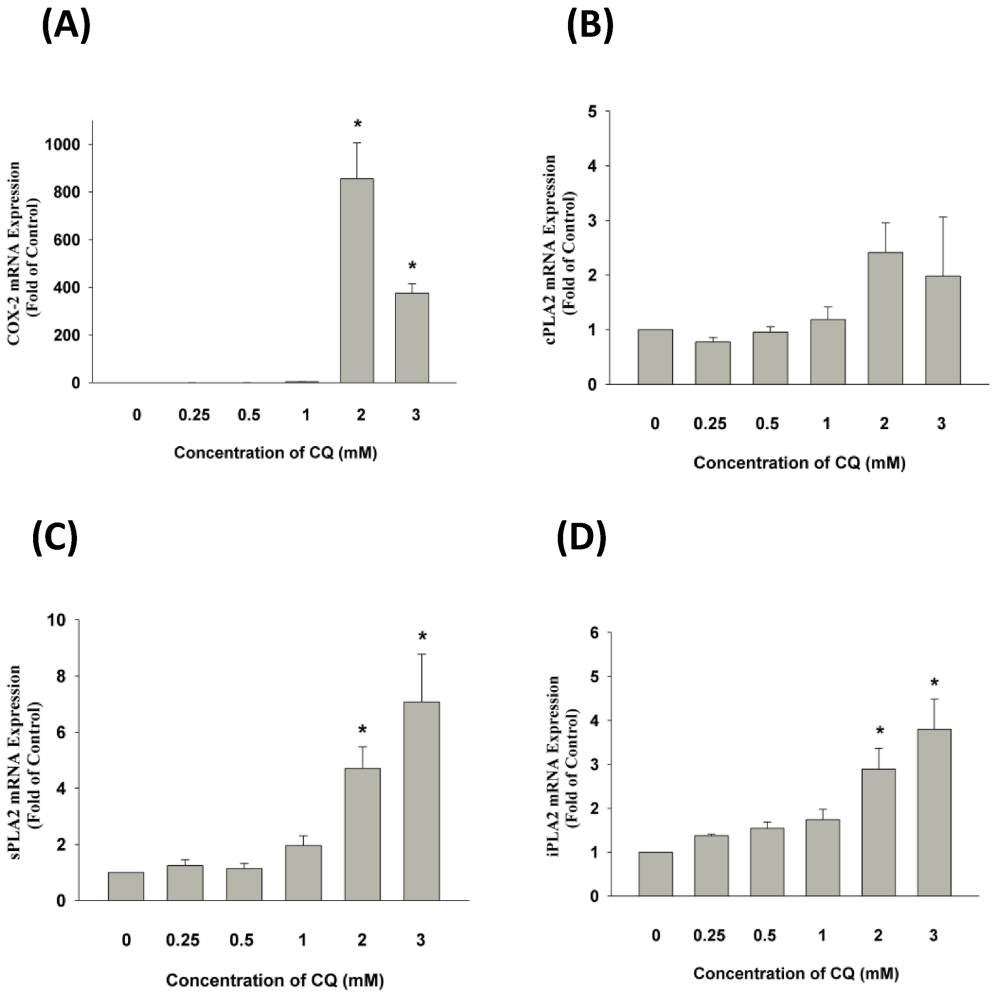

Figure 1 Effect of CQ on the mRNA expression of **(A)** COX-2 (n = 8), **(B)** cPLA2 (n = 20), **(C)** sPLA2 (n = 14), **(D)** iPLA2 (n = 8) of HDPCs as analyzed by real-time PCR. Results were expressed as fold of control (as 1). *denotes statistically significant difference when compared with solvent control (0) group ($P < 0.05$).

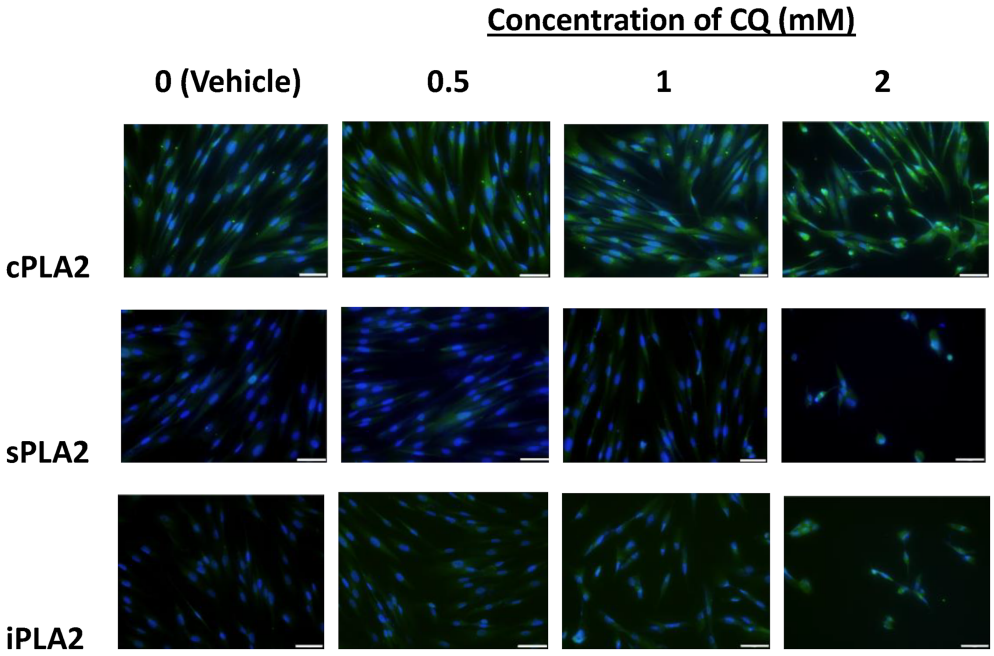

Figure 2 Effect of different concentrations of CQ on the protein expression of cPLA2, sPLA2, and iPLA2 in HDPCs as analyzed by immunofluorescent (IF) staining. One representative IF study result was shown. Blue: DAPI for nuclear staining. Green: staining of target proteins by FITC-conjugated antibody. White scale bar = 50 μm, 400×, original magnification.

Effect of aspirin, eugenol and ASB14780 on camphorqinone-induced cytotoxicity to human dental pulp cells

While aspirin inhibited the CQ-induced PGE2 production, it showed little effect on CQ-induced cytotoxicity to HDPCs. Aspirin at concentrations of 100 and 200 μM showed little effect on cell viability of HDPCs. The CQ-induced cytotoxicity of HDPCs was not affected by aspirin (Fig. 5A). Eugenol at 50 and 100 μM showed no marked effect of cell viability of HDPCs. Eugenol (50 and 100 μM) also showed no obvious influence on cell viability of 2 mM CQ-induced decline of cell viability ($P > 0.05$) (Fig. 5B). Similarly ASB14780 (5 and 10 μM) showed no marked effect on 2 mM CQ-induced cytotoxicity to HDPCs (Fig. 5C).

Discussion

Clinically, the restoration of tooth decay by DBAs and composite resin may potentially induce postoperative sensitivity, pulpal inflammation, and even pulp necrosis especially when the remaining dentin is minimal and resin polymerization is not complete.[1,2] In this study, we found that CQ-induced cPLA2, sPLA2 and iPLA2 expression in HDPCs. Aspirin, eugenol and ASB14780 showed differential effect on CQ-induced 8-isoprostane, PGE2 production and cytotoxicity. These results are useful for clinical operative restorative procedures to protect the dental pulp from chemical-induced injury and inflammation.

Various PLA2 play crucial roles in regulation of cellular inflammatory responses, energy production, fatty acid balance, and generation of lipid barriers.[12] The cPLA2 is crucial enzyme for AA release from membrane store, and mediate the leukotrienes and prostanoids (PGH2, PGD2 etc.) production, and initiate tissue inflammation.[12] PLA2 can be divided into cytosolic PLA2 (cPLA2), secretary PLA2 (sPLA2), iPLA2, etc., with distinct distribution and possible functions and roles in various diseases.[12,19] Among them, the sPLA2, cPLA2 and iPLA2 play critical roles in inflammation and cancer-related diseases.[19] Mitogen-activated protein kinases are shown to phosphorylate and activate cPLA2 (p-cPLA2) to mediate the release of AA from lipid membrane.[20] In the dental pulp, TNFα and IL-1α stimulated COX-2, RANKL, IL-6 etc. in HDPCs, that can be attenuated by cPLA2 inhibitor.[21] TAT & RGD peptide-modified naringin-loaded nanoparticles are also shown to influence the proliferation and differentiation of HDPCs via regulation of sPLA2.[22] Nitric oxide-induced PGE2 production of HDPCs is mediated via phospholipase C, PLA2, and COX-1.[23] Beta-defensin-2 is also reported to induce IL-6, IL-8 and cPLA2 in odontoblast-like cells.[24] All these results suggest the involvement of PLA2 in the inflammation and immune responses of HDPCs. In this study, we found the expression of cPLA2, sPLA2 and moreover iPLA2 in the HDPCs, suggesting their roles in pulpal inflammation and other physiological responses. Besides, Datar et al. reported that CQ significantly altered the metabolism of several important structural lipids in cells at sub-toxic concentrations, which may in turn affect membrane integrity and possibly lead to significant changes in cell responses.[25] However, the influence of various PLA2s and its interaction with CQ on pulpal activities such as regulation of membrane remodeling, bioenergetics, body surface barrier, as well as various cellular biological activities,[12] awaits further investigation.

In this study, we further found that CQ regulated the cPLA2, sPLA2 and iPLA2 expression of HDPCs. The cPLA2

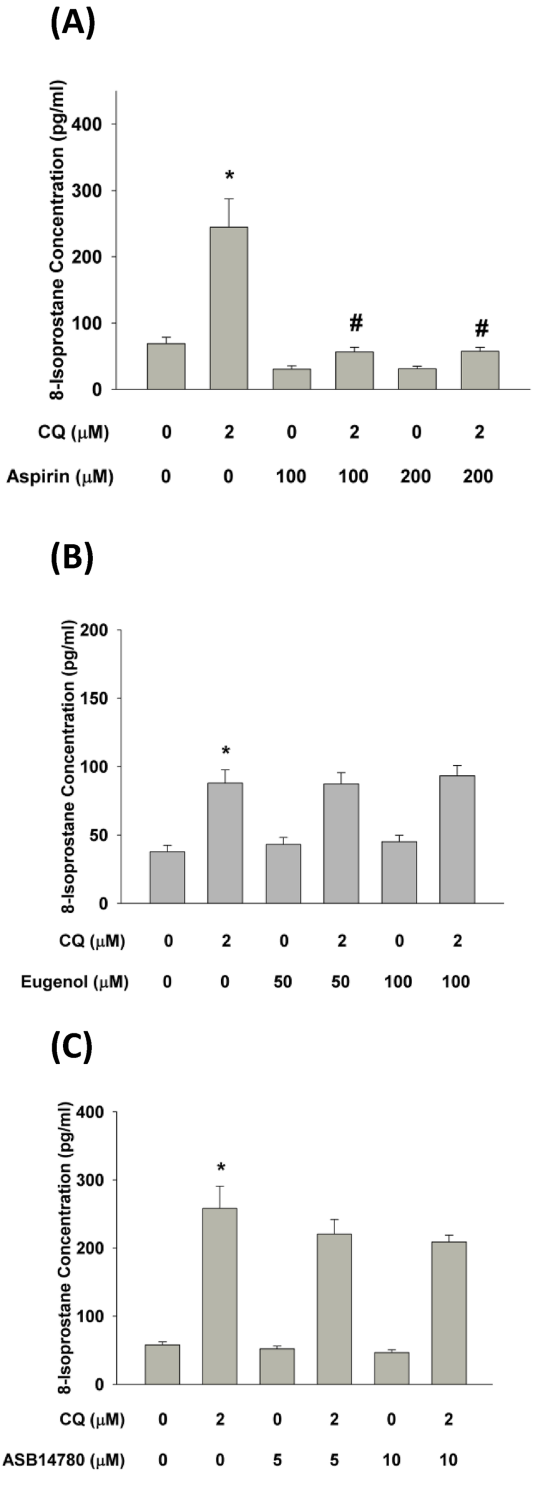

Figure 3 Effect of **(A)** aspirin (n = 5), **(B)** eugenol (n = 6), and **(C)** ASB14780 (n = 10) on CQ-induced 8-isoprostane production of HDPCs as analyzed by ELISA. Results were expressed as pg/ml (Mean ± SE). *denotes statistically significant difference when compared with solvent control (0) group. #indicates statistically significant difference when compared with the CQ-treated group ($P < 0.05$).

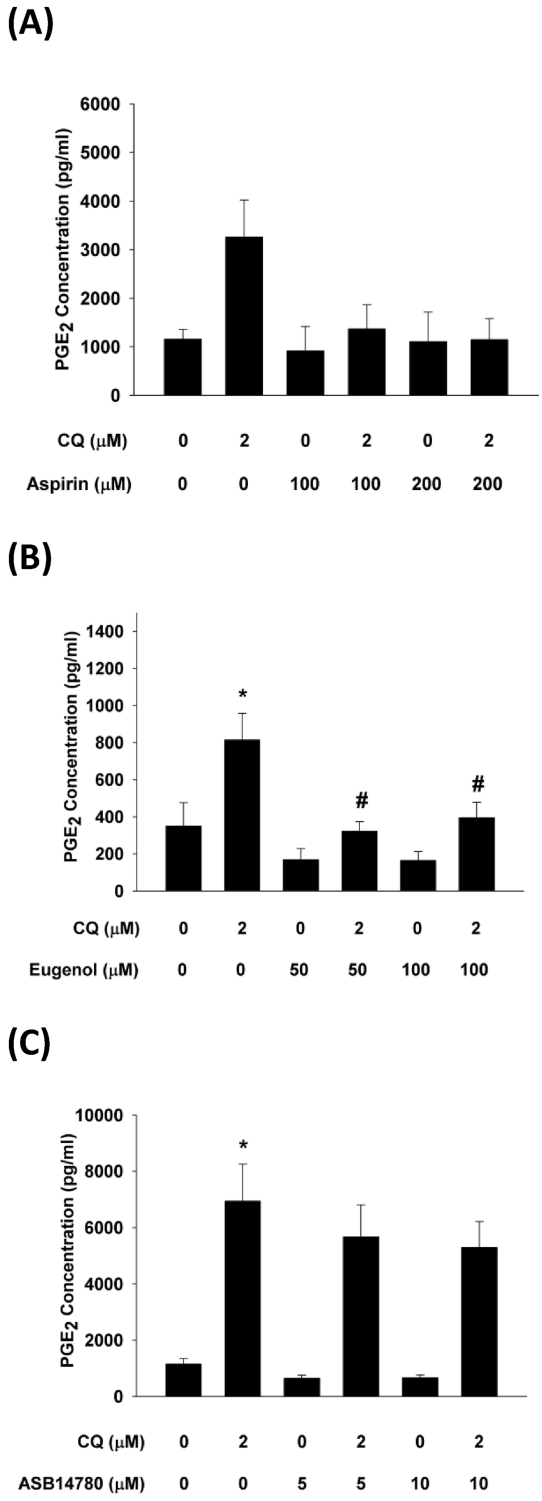

Figure 4 Effect of **(A)** aspirin (n = 16), **(B)** eugenol (n = 10) and **(C)** ASB14780 (n = 11) on CQ-induced PGE_2 production of HDPCs as analyzed by ELISA. Results were expressed as pg/ml (Mean ± SE). *denotes statistically significant difference when compared with solvent control (0) group. #indicates statistically significant difference when compared with the CQ-treated group ($P < 0.05$).

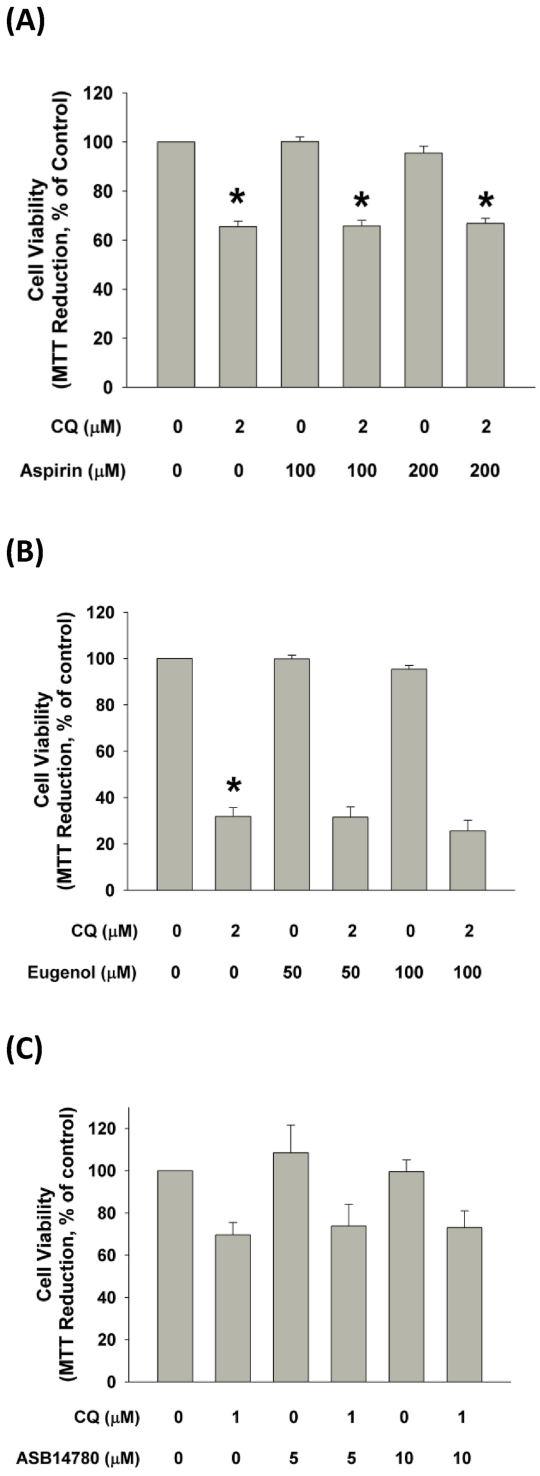

Figure 5 Effect of **(A)** aspirin (n = 9), **(B)** eugenol (n = 11), and **(C)** ASB14780 (n = 5) on CQ (2 mM)-induced cytotoxicity to HDPCs as analyzed by MTT assay. Cell viability results were expressed as percentage of solvent control (as 100 %). *denotes statistically significant difference when compared with solvent control (0) group. #denotes statistically significant difference when compared with CQ-treated group ($P < 0.05$).

family enzymes that are associated with AA, ω3 polyunsaturated fatty acid and other fatty acid metabolism for phosphoinositides, lipid mediators' production, energy generation, cell proliferation and tumorigenicity.[12] Upon activation, cPLA2 is translocated from cytosol to perinuclear Golgi apparatus in response to calcium mobilization.[12] On the other hand, various isoforms of sPLA2 are involved in digestion, immunity, host immune response, inflammation, carcinogenesis and many diseases, possibly via metabolism of AA, phosphatidylcholine, lysophosphatidic acid, prostaglandins, and different fatty acids.[12] iPLA2 plays a role mainly in lipid homeostasis for human life, and involved in several events including lipid mediators' production, triglyceride metabolism, bioenergetics, hepatic steatosis, neurodegeneration, and skin barrier functions.[12] In addition to AA metabolism and inflammatory mediators' release by CQ-induced PLAs in HDPCs, more studies are necessary to clarify the differential role of cPLA2, sPLA2 and iPLA2 in the physiology and pathology such as ROS production and inflammation of human dental pulp.

How to control pulpal inflammation is important to promote pulpal repair and regeneration.[26] In rat experimental pulpitis tissues, increased PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ (a precursor of PGI2) levels were reported,[27] suggesting the involvement of prostaglandins in pulpal inflammation. On the other hand, ROS are involved in many cellular signaling, toxicity events, and mediate a number of physiological and pathological processes such as cancer, tissue inflammation, periodontitis, oral ulcer and mucosal diseases.[28] The rate-limiting step in the formation of PGs (PGE2 and $PGF_{2\alpha}$) from AA is catalyzed by PLA2 and COX. CQ and resin monomers are previously shown to stimulate ROS, 8-isoprostane and PGE2 production as well as cytotoxicity, apoptosis and autophagy.[7,8,11] HDPCs was shown to express prostaglandin EP receptor,[29] to mediate the cellular response to PGE2. Previous studies also found that CQ exert cytotoxicity to different kind of cells at concentrations higher than 1 mM.[5,7] In this study, aspirin (a COX inhibitor), eugenol (a widely-used dental drug with antioxidant, anti-COX, anti-lipoxygenase activity),[30,31] and ASB14780 (a PLA2 inhibitor) were not able to attenuate the CQ-induced cytotoxicity to HDPCs. These results suggest that PGE2 and 8-isoprostane production was not directly mediate CQ-induced cell death. Intriguingly aspirin and eugenol inhibited the CQ-induced PGE2 production, whereas ASB14780 showed only partial inhibition at tested concentrations. Accordingly, aspirin, eugenol and PLA2 inhibitors were reported to suppress PGE2 production by different inducers.[30,32,33] Possibly the clinical intake or local application of COX inhibitor or PLA2 inhibitors can be used to control of pulpal inflammation and pain response.

CQ has been reported to induce lipid peroxidation in erythrocytes.[34] While 8-isoprostane production is generally regarded through free-radical mediated fatty acid (lipid) peroxidation and considered as an oxidative stress marker,[9] some enzymes such as COX, PLA2, prostaglandin H synthase and more others are reported to mediate the peroxidation of lipid and the generation of 8-isoprostane.[35–37] We

unexpectedly found that aspirin and partly also ASB14780, but not eugenol, attenuated the CQ-induced 8-isoprostane production in HDPCs. COX and various phospholipase A2 (PLA2s) have been reported to play important roles in lipid metabolism and lipid peroxidation.[35,36] Aspirin is also shown to induce or inhibit 8-isoprostane production in both in vitro cultured cells and experimental animals.[38–40] This can partly explain why aspirin and ASB14780 showed inhibitory effect toward CQ-induced 8-isoprostane production. On the other hand, only one paper reported the attenuation of 8-isoprostane production in lipopolysaccharide-induced acute lung injury by eugenol in experimental animals.[41] 8-Isoprostance may induce inflammatory effect, provoke airway smooth muscle contraction, as well as stimulate cell proliferation and collagen synthesis of hepatic stellate cells probably through activation of thromboxane A2 receptors.[9,42,43] In gingivitis and periodontitis patients, increased 8-isoprostane levels in gingival crevicular fluid and saliva has been reported.[44,45] Currently, little is known about the biological and pathological effects of 8-isoprostane on the dental pulp and more studies are needed.

In conclusion, CQ may potentially stimulate pulpal inflammation and necrosis possibly via its induction of cytotoxicity, 8-isoprostane, and PGE2 production to the HDPCs, especially when the remaining dentin thickness is minimal. Dental pulp cells may express cPLA2, sPLA2 and iPLA2 that were regulated by CQ. Aspirin, eugenol and PLA2 inhibitors can be possibly used to control of pulpal inflammation after composite resin restoration. However, this study has some limitations such as the use of a single cell type of HDPCs, lack of more mechanistic validation, and the constraints of using *in vitro* models but not also *in vivo* models. More studies are necessary to further confirm the results.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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