Camphorquinone Inhibits Odontogenic Differentiation of Dental Pulp Cells and Triggers Release of Inflammatory Cytokines

Reuben H. Kim, DDS, PhD,†‡ Drake W. Williams, BS,* Susan Bae, BS,* Rachel S. Lee, BS,* Ju-Eun Oh, PhD,* Shebli Mehrazarin, DDS,* Tony Kim, DDS,* Ki-Hyuk Shin, PhD,*†‡ No-Hee Park, DMD, PhD,*†‡§ and Mo K. Kang, DDS, PhD*†‡*

Abstract

Introduction: Camphorquinone (CQ) is a photoinitiator that triggers polymerization of light-curing materials such as dental adhesives and composites. CQ does not become a part of the polymer network, suggesting that CQ can be leached out into surrounding environment including dental pulp and exert adversary effects on tissues. In order to understand the mechanisms of CQ-induced side effects, we investigated the effect of CQ on cell viability, cytokine secretion, and odontogenic differentiation of dental pulp stem cells *in vitro*. **Methods:** Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after CQ exposure. Western blotting was performed for $p16^{INK4A}$, $p21^{WAF1}$, and p53. Secretory cytokines were evaluated using the membrane–enzyme-linked immunosorbent assay as well as conventional and quantitative reverse-transcription polymerase chain reaction. The effects of CQ on odontogenic differentiation were evaluated using alkaline phosphatase and alizarin red S staining methods. **Results:** CQ treatment suppressed the proliferation of DPSCs and induced the expression of $p16^{INK4A}$, $p21^{WAF1}$, and p53. Levels of proinflammatory cytokines (eg, interleukin 6, interleukin 8, and matrix metalloproteinase-3 [MMP3]) were increased by CQ treatment. CQ also inhibited odontogenic differentiation and mineralization capacities of DPSC and MC3T3-E1 cells. **Conclusions:** Our study showed that CQ may trigger pulpal inflammation by inducing proinflammatory cytokine production from the pulpal cells and may impair odontogenic differentiation of dental pulp cells, resulting in pulpal irritation and inflammation. *(J Endod 2013;39:57–61)*

Key Words

Camphorquinone, dental pulp stem cells, inflammation, odontogenic differentiation, pulpal wound healing

Camphorquinone (CQ) is a photoinitiator commonly incorporated in the vast majority of modern dental composite materials including composites and dental adhesives (DAs) (1). CQ uses the visible light-curing systems to initiate the polymerization process (2). When irradiated with visible light in the range of 460–480 nm, CQ generates free radicals, one of the major forms of reactive oxygen species (ROS), in the presence of coinitiators such as tertiary aromatic amines. This free radical initiates the polymerization of monomers such as 2-hydroxyl-ethyl-methacrylate (HEGMA), triethyleneglycol dimethyacrylate, or 2,2-bis(4-[2-hydroxy-3-methacryloxypropoxy] phenyl)propane (3).

Although CQ is one of the important components in dental composite materials, CQ does not become incorporated into the resin polymers, suggesting that there is a likelihood of CQ being leached out into the local environment. CQ is one of the leachable substances in dental materials (4, 5), and it was suggested that the maximum concentration of 14 mmol/L CQ potentially could be leached out into the oral cavity after polymerization (6).

Substances leached out from dental materials were shown to be cytotoxic to cells (7), and several studies have been conducted to examine the cytotoxic effects of CQ on cells *in vitro*. Atsumi et al (8, 9) found that irradiated CQ induced ROS production and inhibited the proliferation of cells. Irradiated CQ also caused DNA damage because of ROS generation as determined using a cell-free system (10). The cytotoxic activity of CQ was shown to be correlated with ROS production (11). Recent studies showed that nonirradiated CQ also has cytotoxic effects, presumably because of its activation in cells by intracellular mechanisms (6). Indeed, nonirradiated CQ was found to generate ROS, leading to DNA alteration in cells (9–13). Collectively, these studies suggest that both irradiated and nonirradiated CQ exert cytotoxic effects.

The cytotoxic effects of CQ on cell viability have been well documented; however, the molecular events associated with the inhibition of cell proliferation or its effects on the differentiation and mineralization potential of dental pulp cells are unknown. In this study, we test the hypothesis that the inhibition of pulp cell proliferation by CQ is linked with an increased expression of cell cycle regulatory genes and inflammatory cytokines.

From *UCLA School of Dentistry, †UCLA Dental Research Institute, ‡UCLA Jonsson Comprehensive Cancer Center, and §David Geffen School of Medicine at UCLA, Los Angeles, California.

Supported in part by the grants (K08DE17121, R03DE30304 to R.H.K., K02DE18959 and R01DE18295 to M.K.K.) from NIDCR/NIH. R.H.K. was supported by the UCLA Academic Senate Faculty Research Grant. M.K.K. was supported by the Jack Weichman Endowment Fund and American Association of Endodontists Foundation grant.

Address requests for reprints to Dr Reuben H. Kim, School of Dentistry, Division of Restorative Dentistry, Center for the Health Sciences, Room 43-091, 10833 Le Conte Avenue, Los Angeles, CA 90095. E-mail address: rkim@dentistry.ucla.edu

0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2012.09.006

Consequently, exposure to CQ impairs the odontogenic differentiation capacity of dental pulp cells.

Materials and Methods

Reagents, Cells, and Cell Culture

Primary dental pulp stem cells (DPSCs) were kindly provided by Dr Songtao Shi (Herman Ostrow School of Dentistry, University of Southern California, Los Angeles, CA). MC3T3-E1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DPSCs and MC3T3-E1 cells were cultured in the basal medium containing 10% fetal bovine serum (FBS) in α-Minimum Essential Medium (MEM) (Invitrogen, Grand Island, NY). To induce these cells to undergo differentiation and mineralization, cells were cultured in the induction medium (IM) that contains 10% FBS, 100 μmol/L ascorbic acid 2-phosphate, 10 mmol/L β-glycerophosphophosphate, and 1.8 mmol/L KH_2PO_4 (Sigma-Aldrich Inc, St Louis, MO) in α-MEM (14). CQ and HEMA were purchased from Sigma-Aldrich and prepared in ethanol.

MTT Assay

Cell viability in response to CQ treatment was accessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Assay (ATCC) according to the manufacturer's protocol. DPSCs were treated with CQ or HEMA for 3 days in the range of 12,500–50 μmol/L. The plates were read at 570 nm using the ELx800 Absorbance Microplate Reader (BioTek, Winooski, VT).

Western Blotting

Cells were lysed and subjected to Western blotting as described previously (14). The following antibodies were used: p53 (DO-1), p21 (C-19), p16 (C-20), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (0411) from Santa Cruz Biotechnology (Santa Cruz, CA).

Conventional and Real-time Quantitative Reverse-transcription Polymerase Chain Reaction

The total RNA was isolated, and complementary DNA was made as described previously (14). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed in triplicates for each sample with LC480 SYBR Green I master (Roche, Indianapolis, IN) using universal cycling conditions on LightCycler 480 (Roche). A total of 45 cycles were executed, and the second derivative Cq value determination method was used to compare fold differences. For the conventional RT-PCR, a total of 35 cycles were executed. The primer sequences are available upon request.

Cytokines Enzyme-linked Immunosorbent Assay Array

The secretory cytokines were detected using the enzyme-linked immunosorbent assay (ELISA)-based Human Antibody Array Assay 3.0 (Panomics, Fremont, CA) according to the manufacturer's protocol. Briefly, 2 mL fresh supernatants collected from DPSCs treated or not with 250 or 500 μmol/L CQ were incubated with the array membrane followed by incubation with biotin-labeled anticytokine antibodies and streptavidin horseradish peroxidase (HRP). The membranes were exposed using chemiluminescence detection method, and the intensity of the dots on the membranes was quantitated using ImageJ software (National Institutes of Health, Bethesda, MD) (15). The dot intensities were normalized to the number of cells at the time harvesting supernatants.

Alkaline Phosphatase Activity and Alizarin Red S Staining

Confluent DPSCs were cultured in IM containing different amounts of CQ for 4 days followed by an additional 6 days without CQ. To stain for alkaline phosphatase (ALP) activity, ALP staining kit (86R-1KT, Sigma-Aldrich Inc) was used according to the manufacturer's protocol. To quantify ALP activity, cells were lysed in lysis buffer (50 mmol/L Tris-HCl [pH = 10], 0.5% Triton X-100 (Sigma-Aldrich Inc), and 1% NP-40). The collected supernatants were incubated with reaction mixture containing 0.2 mol/L glycine-NaOH buffer (pH = 10.4), 16 mmol/L p-NPP substrate (Sigma-Aldrich Inc, Cat #N3254), 1 mol/L $MgCl_2$, and 1 mol/L $ZnCl_2$. The mixture was incubated at 37°C, and the color changes were detected using the microplate reader at 405 nm as described previously (14). Alizarin red S (ARS) staining was performed on DPSCs and MC3T3-E1 cells. Briefly, cells were cultured in IM for 4 weeks and fixed with 1% formalin/phosphate-buffered saline for 10 minutes and stained with 2% ARS solution (pH = 4.1–4.3 with 10% ammonium hydroxide) for 30 minutes at the room temperature. The ARS solution was removed, and cells were washed with ddH_2O. The plates were photographed using a camera. For the quantification of ARS staining, stained cells were destained in 10% cetylpryidinium chloride (Sigma-Aldrich Inc) and measured at 652 nm using the microplate reader. The ImageJ software was also used to quantify the staining (15).

Statistical Analysis

Assays were performed in triplicates (MTT assays, qRT-PCR, or ELISA assay). The results are expressed as the mean ± standard deviation. To compare the outcomes, the Student's *t* test was performed, and *P* values <.05 were considered significant.

Results

CQ Inhibits the Proliferation of DPSCs

To examine the cytotoxic effects of CQ on DPSCs, the MTT assay was performed with various doses for 3 days, and the cell viability was examined. We found that DPSCs started losing their viability between 200 and 400 μmol/L CQ (Fig. 1*A*). As a positive control, we also treated DPSCs with HEMA, which is known to cause apoptosis (16). Compared with HEMA, CQ was more cytotoxic to DPSCs (Fig. 1*A*). When the cells were treated with 250 or 500 μmol/L CQ

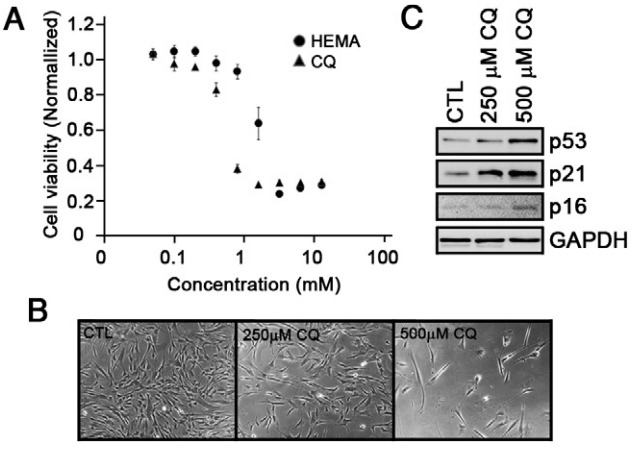

Figure 1. The effects of CQ on proliferation. (*A*) An MTT assay was performed by treating the DPSCs with different doses of CQ or HEMA for 3 days. The experiment was quadruplicated, and the bar represents the standard error. (*B*) Photographs of DPSCs treated with indicated doses of CQ for 3 days were taken (200× magnification). (*C*) CQ-treated DPSCs were harvested and subjected to Western blotting against p53, $p21^{WAF1}$, $p16^{INK4A}$, and GAPDH.