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

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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.

Keywords

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

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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. 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 -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 -glycerophosphosphate, and 1.8 mmol/L KH_2PO_4 (Sigma-Aldrich Inc, St Louis, MO) in -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. 1A). 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. 1A). When the cells were treated with 250 or 500 μmol/L CQ for 3 days, a loss of cell proliferation was noted (Fig. 1B), and it was associated with enhanced expression of p53, p21^{WAF1}, and p16^{INK4A} (Fig. 1C).

CQ Induces Expression of Proinflammatory Cytokines in DPSCs

Next, we sought to determine the expression of cytokines by DPSCs when treatedwith CQ. DPSCs were treated with CQ for 3 days, supernatants were collected, and secreted cytokines were screened using the ELISA-based membrane. Among the 36 cytokines that were screened, we found significant increases in the levels of interleukin (IL)-6, IL-8, and matrix metalloproteinase-3 (MMP3) in CQ-treated DPSCs (Fig. 2A). This was also confirmed using conventional RT-PCR (Fig. 2B) and real-time qRT-PCR (Fig. 2C), indicating that CQ enhances the secretion of IL-6, IL-8, and matrix metalloproteinase-3 (MMP3) in DPSCs.

CQ Inhibits Differentiation and Mineralization Potentials of DPSCs

We examined the effects of CQ on the differentiation potential of DPSCs. In the presence of CQ, the diminution of ALP activity was observed in a dose-dependent manner in DPSCs (Fig. 3A, top panel; Fig. 3B). We also evaluated the mineralization potential and observed reduced ARS staining in the presence of CQ (Fig. 3A, bottom panels; Fig. 3C). To further test whether the effects of CQ on mineralization also occur in other cell types, we used a mouse osteoblastic cell line, MC3T3-E1 cells, and found similar results (Fig. 3D and E). Collectively, these data indicate that CQ inhibits the differentiation and mineralization potentials of dental mesenchymal and osteoblastic cells.

Discussion

The effects of dental material components such as HEMA and triethylene glycol dimethacrylate (TEGDMA) on dental pulp cells are well documented (17). However, there are no reports examining the effects of CQ on the differentiation and mineralization of dental pulp cells. In this study, we showed that CQ inhibits proliferation, enhances the secretion of proinflammatory cytokines, and inhibits the differentiation and mineralization of dental pulp cells *in vitro*. Our study shows the inhibitory effects of CQ on the proliferation and differentiation capacities of human pulp cells, presumably through the induction of inflammatory reaction.

The concentration of CQ in dental resins is found to be approximately 0.17%–1.03% w/w (18), which is equivalent to 3–5 mmol/L. A previous study suggested that the maximum concentration of 14 mmol/L could potentially be leached out into the oral cavity (6). Using CQ at 250 and 500 μmol/L, which is significantly lower than the maximum leachable amounts of CQ, we showed that CQ inhibits the proliferation of DPSCs, which is associated with the induction of p53, p21^{WAF1}, and p16^{INK4A} (Fig. 1). CQ-mediated inhibition of proliferation seems to be primarily caused by p21^{WAF1}, which was significantly induced at 250 μmol/L CQ compared with p53 and p16^{INK4A} (Fig. 1C). p21^{WAF1} is a potent cyclin-dependent kinase inhibitor that inhibits cell cycle progression at the G1 phase (19). In line with this finding, previous cell cycle analysis with CQ revealed significant cell cycle arrest

at the G1 phase (20), suggesting that $p21^{WAF1}$ is the primary mediator that causes arrest of the cell cycle and cell division of dental pulp cells by CQ.

Previous studies suggest that the application of DA as direct pulp capping materials on exposed pulp induces inflammation (21–23). Our study showed that CQ directly induces the secretion of potent proinflammatory cytokines (eg, IL-6, IL-8, and MMP3) (Fig. 2). These cytokines are frequently noted in inflamed pulp tissues (24–26). Therefore, pulpal inflammation is, in part, mediated by CQ in DAs. The differentiation and mineralization of odontogenic cells are other key steps required to complete the pulpal wound healing processes. When treated with various doses of CQ, all the tested cells including DPSC and MC3T3-E1 exhibited the inhibition of differentiation and mineralization (Fig. 3A–E), suggesting that CQ may be one of the components in DA responsible for the improper pulpal wound healing processes *in vivo*.

Previous studies showed that even nonirradiated CQ generated ROS intracellularly and caused DNA damages (6). ROS plays a critical role in the differentiation and mineralization of osteogenic cells. It has been shown that H_2O_2 inhibits osteoblastic differentiation of primary mouse bone marrow stem cells, primary rabbit bone marrow stem cells, and murine osteoblastic cell lines (MC3T3-E1) (27–29). On the other hand, inflammatory cytokines such as IL-6 have been shown to inhibit mineralization both *in vitro* and *in vivo* (30, 31). Therefore, it is possible that CQ-mediated inhibition of mineralization occurs either directly via ROS generation or indirectly via inducing inflammation.

Although our study showed the effects of CQ on the proliferation and differentiation capacities of dental pulp cells, it is likely that other components of dental materials also exert adverse effects on the dental pulp cells *in vivo*. For example, HEMA is known to induce apoptosis (15). The differentiation and mineralization of dental pulp progenitor cells have been recently shown to be inhibited by HEMA and TEGDMA (32). Therefore, the combined effects of these dental materials may be sufficient to suppress pulpal wound healing processes.

Clinically, direct pulp capping with adhesive systems is considered to be not well tolerated because of the induction of pulp inflammation and the lack of dentinal bridge formation even without bacterial presence (33). Our study suggests that CQ may be another contributing factor for inducing such pathological lesions and that the light-curing dental materials containing photoinitiators such as CQ may be not suitable for their use as direct pulp capping materials. Further preclinical and clinical studies warrant a closer examination.

Acknowledgments

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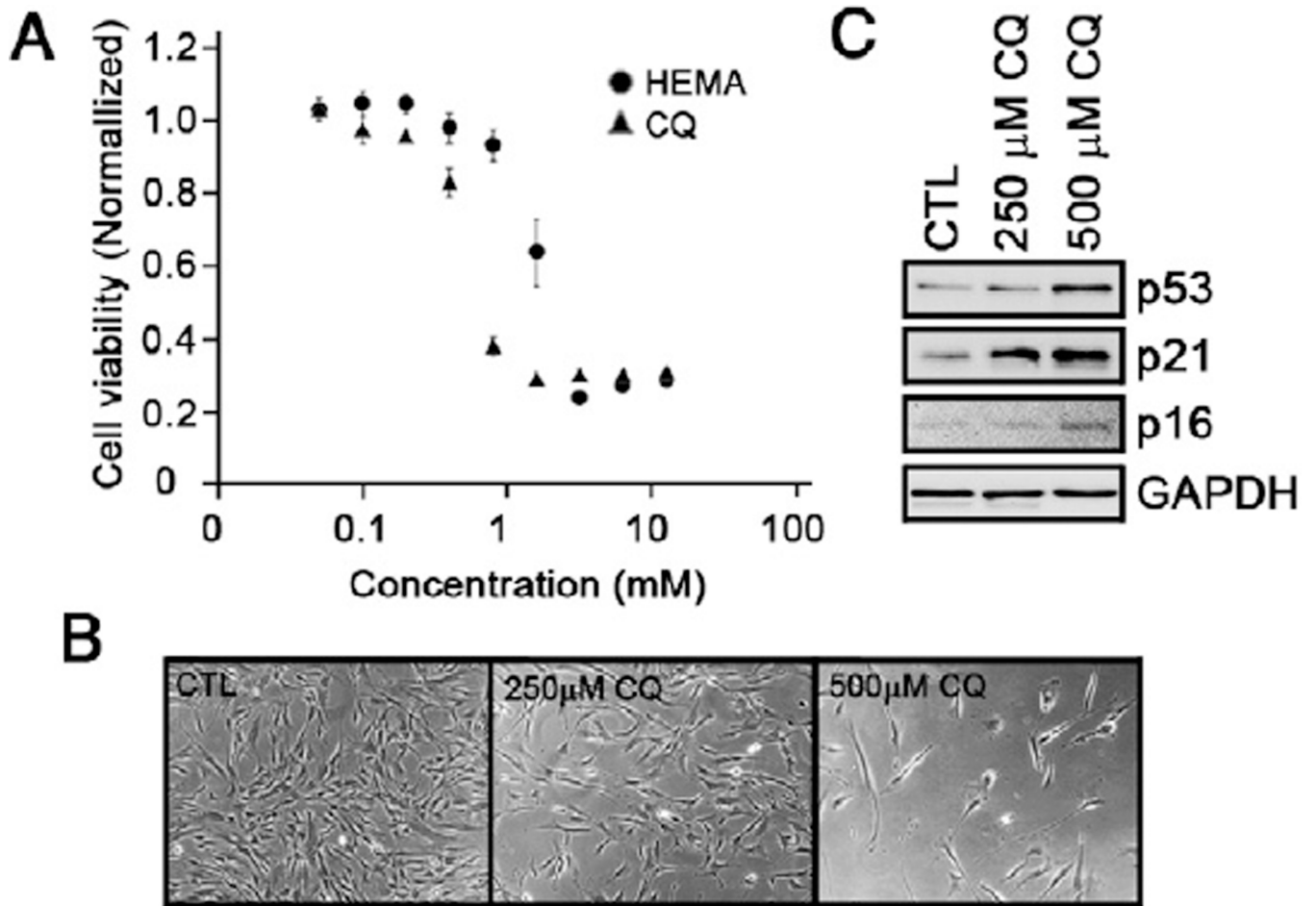
Figure 1.
The effects of CQ on proliferation. (*A*) An MTT assay was performed by treating the DPSCs with different doses of CQ or HEMA for 3 days. The experiment was quadruplicated, and the bar represents the standard error. (*B*) Photographs of DPSCs treated with indicated doses of CQ for 3 days were taken (200× magnification). (*C*) CQ-treated DPSCs were harvested and subjected to Western blotting against p53, p21^{WAF1}, p16^{INK4A}, and GAPDH.
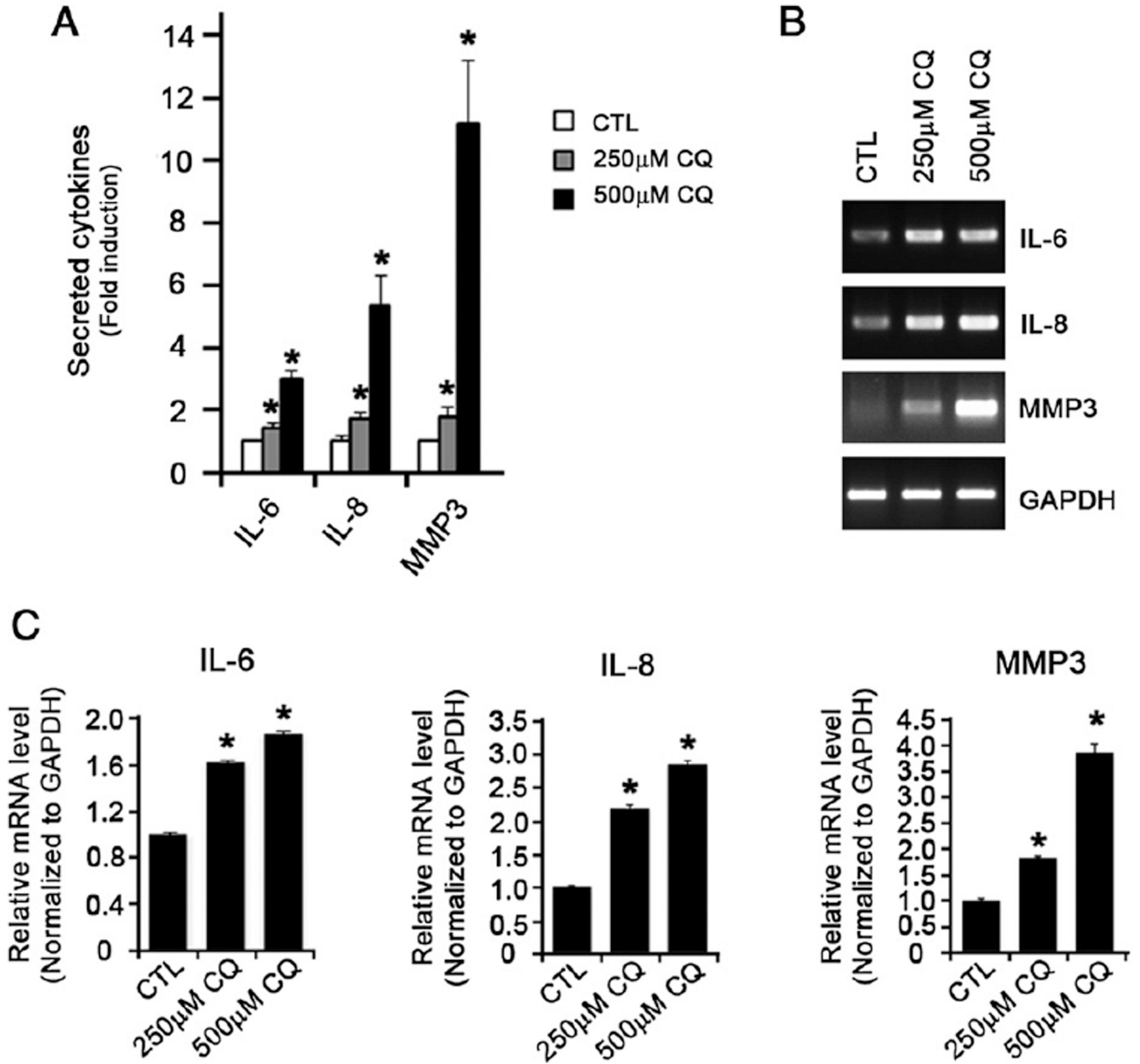
Figure 2.
CQ induces the production of IL-6, IL-8, and MMP3. (*A*) Supernatants from DPSCs treated or not with 250 or 500 μmol/L CQ for 3 days were collected. The secretory cytokines were detected using ELISA-based Human Antibody Array Assay 3.0. The intensity of the dots indicating the presence of corresponding cytokines was quantified using ImageJ software. The fold induction was indicated in relation to the intensity from the CQ-untreated cells. (*B*) The same cells were collected and subjected to conventional RT-PCR. (*C*) Real-time qRT-PCR was also performed in triplicate. GAPDH was used to normalize.
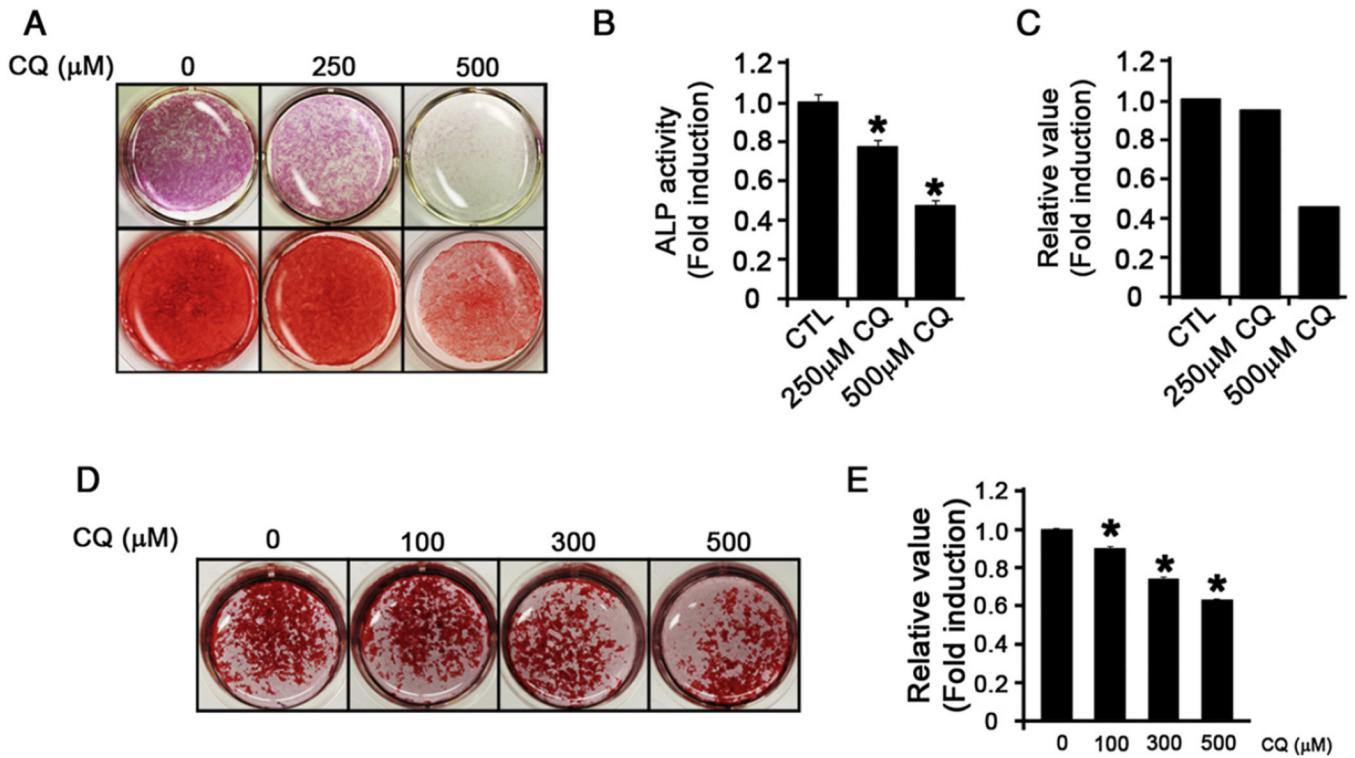
Figure 3.
CQ inhibits the differentiation and mineralization of DPSCs. (*A*) DPSCs treated or not with CQ in IM were cultured for 10 days or 4 weeks, and the differentiation or mineralization potentials were evaluated using ALP (top panels) or ARS staining (bottom panels) methods, respectively. (*B*) The ALP activity in CQ-treated DPSCs was quantified using a microplate reader at 405 nm. (*C*) ARS staining was quantified using ImageJ software. (*D*) A similar experiment was performed using MC3T3-E1 cells, and the ARS staining was performed to examine the mineralization potential. (*E*) ARS staining was destained and quantified using a microplate reader at 652 nm.