Coenzyme Q_0 Enhances Ultraviolet B–Induced Apoptosis in Human Estrogen Receptor–Positive Breast (MCF-7) Cancer Cells

Hui-Min Wang, PhD[1], Hsin-Ling Yang, PhD[2], Varadharajan Thiyagarajan, PhD[2], Tzu-Hsiang Huang, MS[2], Pei-Jane Huang, PhD[3], Ssu-Ching Chen, PhD[4], Jer-Yuh Liu, PhD[2], Li-Sung Hsu, PhD[5], Hsueh-Wei Chang, PhD[1,6], and You-Cheng Hseu, PhD[2,3]

Abstract

Coenzyme Q_0 (CoQ$_0$; 2,3-dimethoxy-5-methyl-1,4-benzoquinone), a major active constituent of *Antrodia camphorata*, has been shown to inhibit human triple-negative breast cancer (MDA-MB-231) cells through induction of apoptosis and cell-cycle arrest. Ecological studies have suggested a possible association between ultraviolet B (UVB) radiation and reduction in the risk of breast cancer. However, the underlying mechanism of the combination of CoQ$_0$ and UVB in human estrogen receptor–positive breast cancer (MCF-7) remains unclear. In this study, the possible effect of CoQ$_0$ on inducing apoptosis in MCF-7 cells under exposure to low-dose UVB (0.05 J/cm^2) has been investigated. CoQ$_0$ treatment (0-35 μM, for 24-72 hours) inhibits moderately the growth of breast cancer MCF-7 cells, and the cell viability was significantly decreased when the cells were pretreated with UVB irradiation. It was noted that there was a remarkable accumulation of subploid cells, the so-called sub-G1 peak, in CoQ$_0$-treated cells by using flow cytometric analysis, which suggests that the viability reduction observed after treatment may result from apoptosis induction in MCF-7 cells. CoQ$_0$ caused an elevation of reactive oxygen species, as indicated by dichlorofluorescein fluorescence, and UVB pretreatment significantly increased CoQ$_0$-induced reactive oxygen species generation in MCF-7 cells. In addition, cells were exposed to CoQ$_0$, and the induction of DNA damage was evaluated by single-cell gel electrophoresis (comet assay). CoQ$_0$-induced DNA damage was remarkably enhanced by UVB pretreatment. Furthermore, CoQ$_0$ induced apoptosis in MCF-7 cells, which was associated with PARP degradation, Bcl-2/Bax dysregulation, and p53 expression as shown by western blot. Collectively, these findings suggest that CoQ$_0$ might be an important supplemental agent for treating patients with breast cancer.

Keywords

CoQ$_0$, breast cancer, ultraviolet B, ROS, apoptosis, chemotherapy

Submitted Date: 29 April 2016; Revised Date: 27 August 2016; Acceptance Date: 13 September 2016

Introduction

Breast cancer is the leading cause of cancer death for women in Taiwan.[1] The incidence rate of breast cancer among Chinese women has increased enormously with changes in lifestyle. Although incidence of breast cancer is increasing worldwide, the mortality rate has declined in developed countries as a result of a range of measures, including implementation of screening and introduction of adjuvant systemic treatments.[2,3] Studies reveal that about two thirds of breast cancer patients have estrogen-receptor (ER)–dependent tumors and most patients are treated with

[1]National Chung Hsing University, Taichung, Taiwan
[2]China Medical University, Taichung, Taiwan
[3]Asia University, Taichung, Taiwan
[4]National Central University, Chung-Li, Taiwan
[5]Chung Shan Medical University, Taichung, Taiwan
[6]Kaohsiung Medical University Hospital, Kaohsiung, Taiwan

Corresponding Author:
You-Cheng Hseu, Department of Cosmeceutics, College of Biopharmaceutical and Food Sciences, China Medical University, Taichung 40402, Taiwan.
Email: ychseu@mail.cmu.edu.tw

tamoxifen (an ER antagonist) and aromatase inhibitors, which abates estrogen activity.[4] However, concerns about the safety and effectiveness of hormone therapy still exist due to side effects that substantially affect the patient's quality of life. Therefore, it is necessary to explore more efficient potential therapeutic strategies to reduce the risk of breast cancer.[5]

Ultraviolet (UV) radiation is a part of the sunlight spectrum, which can be categorized into 3 bands—UVA (320-400 nm), UVB (280-320 nm), and UVC (100-280 nm)—according to wavelength.[6] Among these, DNA is the best-known target of UVB.[7] Low doses of UVB cause DNA mutations, which eventually lead to tumor initiation, whereas high doses result in irreparable DNA damage, causing apoptosis and cell death.[8] The presence of singlet oxygen in apoptosis has also been reported in UVB-irradiated retinal pigment epithelial cells.[9] A combined use of UVB irradiation and arsenic treatment exhibits the antiproliferative and pro-apoptotic effects via activation of caspase-8 and -9 in keratinocytes. In addition, it has also been reported that UVB radiation efficiently increased the apoptosis in an ER-positive breast cancer cell line.[10]

Antrodia camphorata, an indigenous medicinal mushroom, has been used in traditional Chinese medicine for protection of diverse health conditions such as muscle damage, fever, food poisoning, drug intoxication, diarrhea, hypertension, skin irritation, and cancer.[11] Accumulating evidence suggests that *Antrodia camphorata* possesses a broad range of biological activities, including antioxidant, anticancer, antihyperlipidemic, immunomodulatory, antimetastasis, hepatoprotective, antihypertensive, and anti-inflammatory properties.[11,12] The mycelium and the fruiting bodies of *Antrodia camphorata* exerted strong anticancer activity against several cancers such as breast, liver, prostate, colon, bladder, oral, pancreatic, lung, and leukemic cells. The inhibitory effect against cancer cells by *Antrodia camphorata* could be mediated by different cellular mechanisms of actions, such as inhibition of metastasis and angiogenesis regulatory proteins; regulation of oncogene and tumor suppressor gene expression; downregulation of signal transduction cascades involving Nrf2, NF-κB, AP-1, and MAPK; induction of cell-cycle arrest; and apoptosis involving the Wnt/β-catenin, p53, death ligands, Bcl-2, and caspase families.[11,13,14] According to our previous results of high-performance liquid chromatography analysis, the amount of CoQ_0 in the fermented culture broth of *Antrodia camphorata* was 17.3% (254 nm).[15]

Coenzyme Q_0 (CoQ_0 or Ubiquinone 0) is a redox-active ubiquinone compound that accumulates predominantly in mitochondria. Recently, we have reported the anti-angiogenic and anti-inflammatory properties of CoQ_0 in vitro or in vivo.[16,17] Several studies suggest that CoQ_0 exhibits strong toxicity toward various cancer cell lines.[18,19] CoQ_0 treatment also was shown to decrease the cell proliferation in HepG2,

A549, and SW480 cancer cell lines;[18] stimulate insulin secretion in pancreatic islets;[20] possess anti-angiogenic properties;[16] and inhibit oxidative damage in mouse blood and tissues. Despite its cytotoxicity, some in vivo studies exhibited no deleterious effects of a CoQ_0 analog in combination with other nutrients. Notably, administration of CoQ_0 mixture inhibited oxidative damage in blood, heart, liver, kidney, and spleen of rodents.[21,22] Nevertheless, pharmacological activities of a single CoQ_0 molecule against cancer and redox imbalance have not been fully studied, and precise signaling pathways involved are largely unknown.

Accumulating evidence suggests that many natural compounds from food and plants have chemotherapeutic and chemopreventive effect in several human cancers.[23,24] A number of natural products extracted from Chinese herbs has been found to enhance chemotherapy by inducing apoptosis and exhibiting anticancer potential both in vitro and in vivo.[25-27] These studies indicate effects of CoQ_0 on anticancer activity against human triple-negative breast (MDA-MB-231) cancer cells through induction of apoptosis and cell-cycle arrest.[19] In our previous study, we demonstrated that CoQ_0, a major active constituent of AC, significantly inhibited melanoma cell growth through the induction of cell-cycle arrest and apoptosis via Wnt/β-catenin signaling pathways.[28] Studies have suggested a possible association between UVB radiation and reduction in the risk of breast cancer.[29] However, the regulatory mechanisms of CoQ_0 that produces its pro-apoptosis effects in MCF-7 breast cancer are unknown. In the current study, the effect of CoQ_0 treatment alone and in combination with UVB has been examined on the cellular growth of MCF-7 breast cancer cells.

Materials and Methods

Reagents and Antibodies

CoQ_0 (2,3 dimethoxy-5-methyl-1,4 benzoquinone) was purchased from Sigma-Aldrich (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin/neomycin were obtained from GIBCO BRL/Invitrogen (Carlsbad, CA). p53, Bcl-2, and β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc (Heidelberg, Germany). PARP antibody was obtained from Cell Signaling Technology, Inc (Danvers, MA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich Chemical Co (St Louis, MO). CoQ_0 was dissolved in dimethyl sulfoxide (DMSO) and diluted with DMEM to make the final concentration below 0.01%. All other chemicals were of the highest grade commercially available and were supplied either by Merck or Sigma.

Cell Culture

The estrogen receptor–positive MCF-7 (human breast adenocarcinoma) cell line was obtained from the Bioresource Collection and Research Center (BCRC, Taiwan). MCF-7 cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 1% penicillin-streptomycin-neomycin in a humidified incubator (5% CO_2 in air at 37°C). Cultures were harvested and monitored for cell number by counting cell suspensions with a hemocytometer. Cell morphology was examined using phase-contrast microscopy (200× magnification).

UVB Irradiation and Sample Treatment

Prior to UVB irradiation, MCF-7 cells were washed with phosphate-buffered saline (PBS) and resuspended in fresh phenol red–free DMEM containing 1% FBS. Then, cells were exposed to UVB radiation at dose 0.05 J/cm^2 (λ_{max}, 312 nm; no detectable emission below 280 nm) using UVllink CL-508M (UVItec, Cambridge, UK) for 30 seconds. After UVB irradiation, the cells were treated with CoQ_0 (0-35 μM) for 72 hours in DMEM containing 10% FBS.

Assessment of Cell Viability by MTT Assay

Cell viability was determined by the MTT colorimetric assay. MCF-7 cells (5 × 10^4 cells/well in 24-well plates) were treated with various concentrations of CoQ_0 (0-35 μM) for 24 to 72 hours, before 400 μL 0.5 mg/mL MTT in PBS was added to each well. After incubation at 37°C for 2 hours, an equal volume of DMSO (400 μL) was added to dissolve the MTT formazan crystals, and the absorbance was measured at 570 nm (A_{570}) using an ELISA microplate reader (μ-Quant, Winooski, VT). The percentage of cell viability was calculated as follows: (A_{570} of treated cells/A_{570} of untreated cells) × 100.

Flow Cytometric Analysis

Cellular DNA content was determined by flow cytometry using the propidium iodide (PI)–labeling method as described previously.[14] Briefly, MCF-7 cells were seeded at a density of 1 × 10^6 cells/dish in 10-cm dishes. After UVB (0.05 J/cm^2) irradiation, cells were treated with increasing concentrations of CoQ_0 (0-35 μM) for 72 hours. Cells were harvested, fixed in ice-cold 70% ethanol, stored at 4°C, washed with PBS (pH 7.2), treated with 25 μg/mL RNase A at 37°C for 15 minutes, and stained with 50 μg/mL PI for 20 minutes. Flow cytometric profiles were analyzed with ModFit software (Verity Software House, Topsham, ME). Apoptotic nuclei were identified as a subploid DNA peak and were distinguished from cell debris on the basis of forward light scatter and PI fluorescence.

Measurement of Reactive Oxygen Species Generation

Intracellular reactive oxygen species (ROS) accumulation was detected by fluorescence microscopy using the cell-permeable fluorogenic probe DCFH$_2$-DA method. Cells (1 × 10^5 cells/12-well) were cultured in DMEM medium that had been supplemented with 10% FBS, and the culture medium was replaced when the cells had reached 80% confluence. After UVB (0.05 J/cm^2) irradiation, cells were treated with CoQ_0 (35 μM) for 20 minutes. Then culture supernatants were removed, and incubated with nonfluorescent DCFH$_2$-DA (10 μM) in fresh medium at 37°C for 30 minutes. ROS production was measured by changes in fluorescence due to the intracellular accumulation of dichlorofluorescein (DCF), which is caused by the oxidation of DCFH$_2$. The DCF fluorescence stained cells were captured using fluorescence microscope (200× magnification; Olympus, Center Valley, PA). The fluorescence intensity was quantified using analysis LS 5.0 soft image solution (Olympus Imaging America Inc). The fold-increase of ROS generation was compared with the vehicle-treated cells, which were arbitrarily considered as 1-fold.

Comet Assay

The comet assay (single-cell gel electrophoresis assay) is an uncomplicated and sensitive technique for the detection of DNA damage at the level of individual eukaryotic cells.[30] The protocol used for the comet assay followed previously published guidelines.[31] After UVB (0.05 J/cm^2) irradiation, MCF-7 cells (1 × 10^6 cells/10-cm dish) were treated with CoQ_0 (35 μM) for 72 h. Cells were then suspended in 1% low-melting-point agarose in PBS (pH 7.4) and pipetted onto superfrosted glass microscope slides that had been precoated with a layer of 1% normal-melting-point agarose (which was warmed to 37°C prior to use). The agarose was allowed to set at 4°C for 10 minutes; then, the slides were immersed in lysis solution (containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% Triton X-100) at 4°C for 1 hour. The slides were then placed in single rows inside a 30-cm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA (pH 13.4) at 4°C for 40 minutes to allow the separation of the 2 DNA strands (alkaline unwinding). Electrophoresis was performed in the unwinding solution at 30 V (1 V/cm), 300 mA, for 30 minutes. The slides were then washed 3 times for 5 minutes each with 0.4 M Tris (pH 7.5) at 4°C before they were stained with DAPI (1 μg/mL). Observations were made at a final magnification of 200× using a UV microscope with a 435 nm excitation filter. Visual scoring of the cellular DNA on each slide was based on the characterization of 100 randomly selected nucleoids. Comet-like DNA formations were categorized into 5 classes (0, 1, 2, 3, or 4) representing increasing DNA damage in the

form of a "tail." Each comet was assigned a value according to its class. The overall score for 100 comets ranged from 0 (100% of comets in class 0) to 400 (100% of comets in class 4), and the overall DNA damage in the cell population was expressed in arbitrary units.[32] The observation and analysis of the results were always performed by the same experienced observer. The observer was blinded and had no knowledge of the slide identity. DNA damage in cells, which was specified as DNA strand breaks including double and single-strand variants at alkali-labile sites, was analyzed under alkaline conditions (pH 13.4). DNA migration from the nucleus of each cell was measured with a computer program using the comet moment parameters as follows: comet moment = $\sum 0 \rightarrow n$ [(amount of DNA at distance X) × (distance X)]/total DNA. The tail moments were quantified using the Cometscore software (Sumerduck, VA).

Protein Isolation and Western Blot Analysis

After UVB (0.05 J/cm^2) irradiation, MCF-7 cells (1×10^6 cells/10-cm dish) were incubated with CoQ$_0$ (35 µM) for 72 hours. After incubation, the cells were harvested, pooled, washed once with PBS, and suspended in 100 µL of lysis buffer (10 mM Tris-HCl, pH 8, 320 mM sucrose, 1% Triton X-100, 5 mM EDTA, 2 mM DTT, and 1 mM PMSF). The cell lysates were maintained on ice for 20 minutes and then centrifuged at $15\,000 \times g$ for 30 minutes at 4°C. Total protein content was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA), BSA as a standard. The protein extracts were mixed with sample buffer (62 mM Tris-HCl, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol), and the mixture was boiled at 97°C for 5 minutes. Equal amounts (50 µg) of denatured protein samples were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and then transferred onto polyvinylidene difluoride membranes overnight. The membranes were blocked with 5% nonfat dried milk in PBS containing 1% Tween-20 for 1 hour at room temperature, followed by incubation with primary antibodies overnight. The membranes were then incubated with either a horseradish peroxidase–conjugated anti-rabbit or anti-mouse antibodies for 2 hours prior to development using a chemiluminescent substrate (Millipore, Billerica, MA). Densitometric analyses were performed using commercially available quantitative software (AlphaEase, Genetic Technology Inc, Miami, FL), with the control representing 1.0-fold.

Statistical Analyses

The obtained data were analyzed using analysis of variance (ANOVA), followed by Dunnett's test for pairwise comparison. Mean values are presented with their standard deviation (mean ± SD). Statistical significance between the treatments was defined as $P < .05$ for all tests.

Results

CoQ$_0$ Enhances UVB-Induced Cell Death in MCF-7 Cells

Prior to investigating the apoptotic effects of CoQ$_0$, the cytotoxic effects of CoQ$_0$ on human breast cancer MCF-7 cells were examined using an MTT colorimetric assay. The cells were exposed to 0 to 35 µM of CoQ$_0$ for 24 to 72 hours. As shown in Figure 1, treating MCF-7 cells with 35 µM CoQ$_0$ for 24 and 48 hours resulted in no significant effect on cell viability. However, cell viability dramatically decreased when the MCF-7 cells were treated with CoQ$_0$ (35 µM) for 72 hours (Figure 1A-C).

In addition, CoQ$_0$-enhanced cell death was assessed by pretreating the MCF-7 cells with UVB (0.05 J/cm^2) for 72 hours. We observed that exposure to UVB (0.05 J/cm^2) dramatically induced MCF-7 cell death, which was significantly increased by CoQ$_0$ (35 µM, Figure 1D). These data further confirm that UVB-induced cell damage was remarkably enhanced by CoQ$_0$ (35 µM). The sublethal concentration of CoQ$_0$ (35 µM) were used for subsequent experiments.

CoQ$_0$ Increases Apoptosis in UVB-Irradiated MCF-7 Cells

Flow cytometric analyses were conducted to assess the apoptotic levels in CoQ$_0$ (0-35 µM) and UVB (0.05 J/cm^2)-irradiated MCF-7 cells by measuring the fluorescence of the PI-DNA complex. The results showed that treatment of MCF-7 cells with CoQ$_0$ dose-dependently increased the sub-G1 peak as compared to control (Figure 2A and C). Furthermore, UVB pretreatment possessed a greater ability to induce sub-G1 peak in MCF-7 cells than CoQ$_0$ and UVB alone (Figure 2B and D). These data further suggest that CoQ$_0$ treatment may enhance UVB-induced apoptosis in MCF-7 cells.

CoQ$_0$ Induces Intracellular ROS Generation in UVB-Irradiated MCF-7 Cells

ROS generation has been implicated as an early event during apoptosis. To determine whether CoQ$_0$-induced apoptosis is ROS-dependent, we examined the involvement of CoQ$_0$ in ROS generation in UVB-irradiated MCF-7 cells. Fluorescence spectroscopy using DCFH$_2$-DA as a fluorescent probe was performed to estimate intracellular ROS generation. The results indicated that MCF-7 cells exposed to CoQ$_0$ (35 µM) for 20 minutes showed significant increase of fluorescence, which is proportionate to the amount of ROS generation. In addition, UVB irradiation markedly increased the intracellular ROS level as compared to control. Interestingly, CoQ$_0$ treatment significantly enhanced the ROS generation in the UVB-irradiated cells (Figure 3).
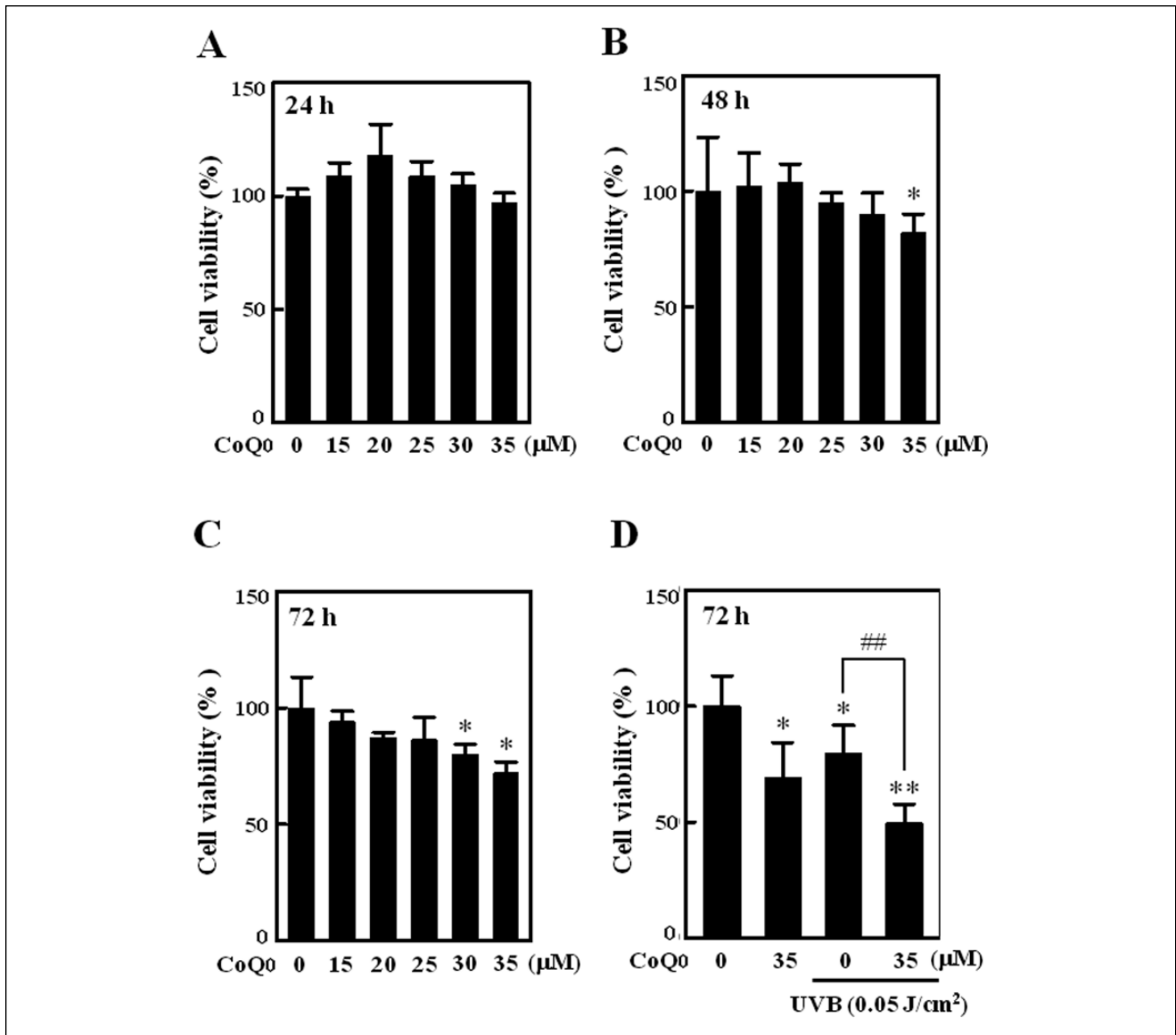
Figure 1. CoQ_0 treatment significantly enhances UVB-mediated inhibition of MCF-7 cell growth. (A-C) Cells were treated with various concentrations of CoQ_0 (0-35 μM) for 24 to 72 hours. (D) Cells were irradiated with UVB (0.05 J/cm^2) and then treated with or without CoQ_0 (0-35 μM) for 72 hours. The effects of CoQ_0 alone and along with UVB were determined by MTT assay. The results are the mean ± SD of 3 assays. *$P < .05$; **$P < .01$; ***$P < .001$ compared with untreated control cells. #$P < .05$; ##$P < .01$; ###$P < .001$ compared with UVB-irradiated cells.

These data suggest that induction of excessive ROS formation by CoQ_0 treatment may contribute to mitochondrial dysfunction and DNA damage in UVB-irradiated MCF-7 cells.

CoQ_0 Enhances UVB-Induced DNA Damage in MCF-7 Cells

DNA damage, represented by DNA single-strand breaks, was indicated by an increase in tail lengths. DNA damage was evaluated with the comet assay, in which the tail length is an important quantitative parameter. Therefore, the effect of CoQ_0 treatment (35 μM for 72 hours) on cellular DNA damage was evaluated in a single-cell gel electrophoresis comet assay. A total toxicity scale was generated while considering the comet lengths. Our results showed that a significant increase in comet length was observed after MCF-7 cells were treated with CoQ_0 for 72 hours (Figure 4A and B). Furthermore, comet length and tail moment were dramatically increased when the cells were pretreated with UVB irradiation (Figure 4C and D), clearly indicating that CoQ_0 treatment enhanced UVB-induced DNA damage.
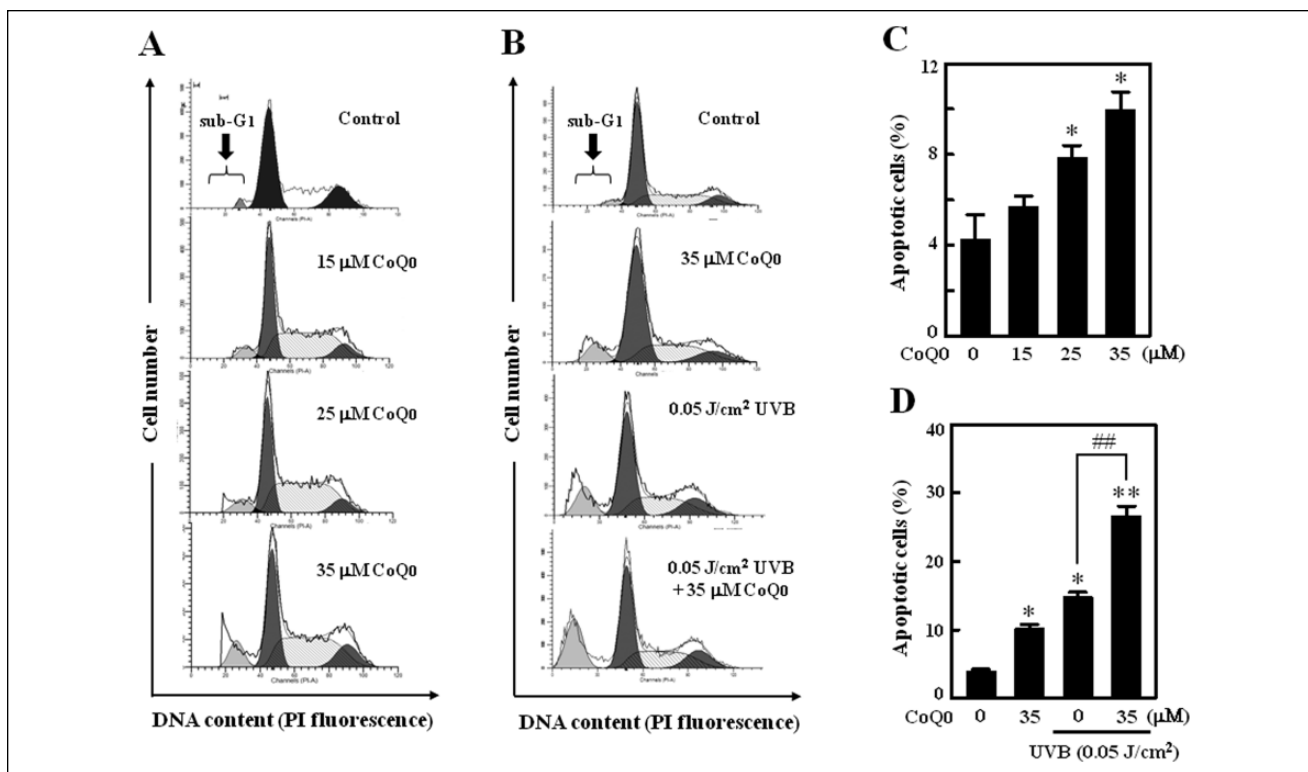
Figure 2. CoQ_0 treatment significantly enhances sub-G1 peak in UVB-irradiated MCF-7 cells. (A) Cells were treated with various concentrations of CoQ_0 (0-35 μM) for 72 hours. (B) Cells were irradiated with UVB (0.05 J/cm^2) and then treated with or without CoQ_0 (0-35 μM) for 72 hours. Cellular DNA content was determined by flow cytometry using the PI-labeling method as described in Materials and Methods. (C and D) The histograms indicate the percentage of apoptotic-positive cells induced by CoQ_0 and UVB. The results are the mean ± SD of 3 assays. *P < .05; **P < .01; ***P < .001 compared with untreated control cells. #P < .05; ##P < .01; ###P < .001 compared with UVB-irradiated cells.

CoQ_0 Upregulates p53 and PARP Cleavage in MCF-7 Cells

The p53 tumor-suppressor protein plays an important role in the induction of apoptosis.[33] Therefore, we examined p53 expression to determine the possible role of p53 in the induction of apoptosis by CoQ_0 and UVB in MCF-7 cells. Western blot analyses revealed that pretreatment of MCF-7 cells with UVB significantly increased the p53 expression compared to CoQ_0 and UVB alone (Figure 5A).

In addition, the cleavage of PARP, a nuclear enzyme that is associated with DNA repair, is dependent on the proteolytic cleavage of caspase-3 and is considered to be an important biological characteristic of apoptosis. As shown in Figure 5B, the PARP (115 kDa) protein was cleaved into an 85 kDa fragment in MCF-7 cells in response to CoQ_0 treatment. Furthermore, UVB pretreatment significantly increased the PARP cleavage as compared to control. Taken together, these data suggest that CoQ_0-induced apoptosis in UVB-irradiated MCF-7 cells was accompanied by p53 activation and PARP degradation.

CoQ_0 Dysregulates Bcl-2/Bax Expression in UVB-Irradiated MCF-7 Cells

Bcl-2 family proteins play a pivotal role in (mitochondria-mediated) apoptosis, either as activators (Bok, Bad, and Bax) or as inhibitors (Bcl-2, Bcl-xL, and Bcl-w).[34] The effect of CoQ_0 and UVB on Bcl-2 and Bax protein levels were examined in MCF-7 cells. As shown in Figure 5C, CoQ_0 and UVB caused a dramatic reduction in Bcl-2, and significant increase of Bax protein levels was observed. Interestingly, the combination of UVB and CoQ_0 significantly increased the Bcl-2 and Bax protein ratio as compared to control. These results indicate that CoQ_0 may alter the Bcl-2 and Bax protein ratio and induce apoptosis in UVB-irradiated MCF-7 cells.

Discussion

Breast cancer is the most common cancer among women and is the second leading cause of cancer-related death worldwide.[35] The inhibition of proliferation and the induction of apoptosis are currently being employed as criteria by
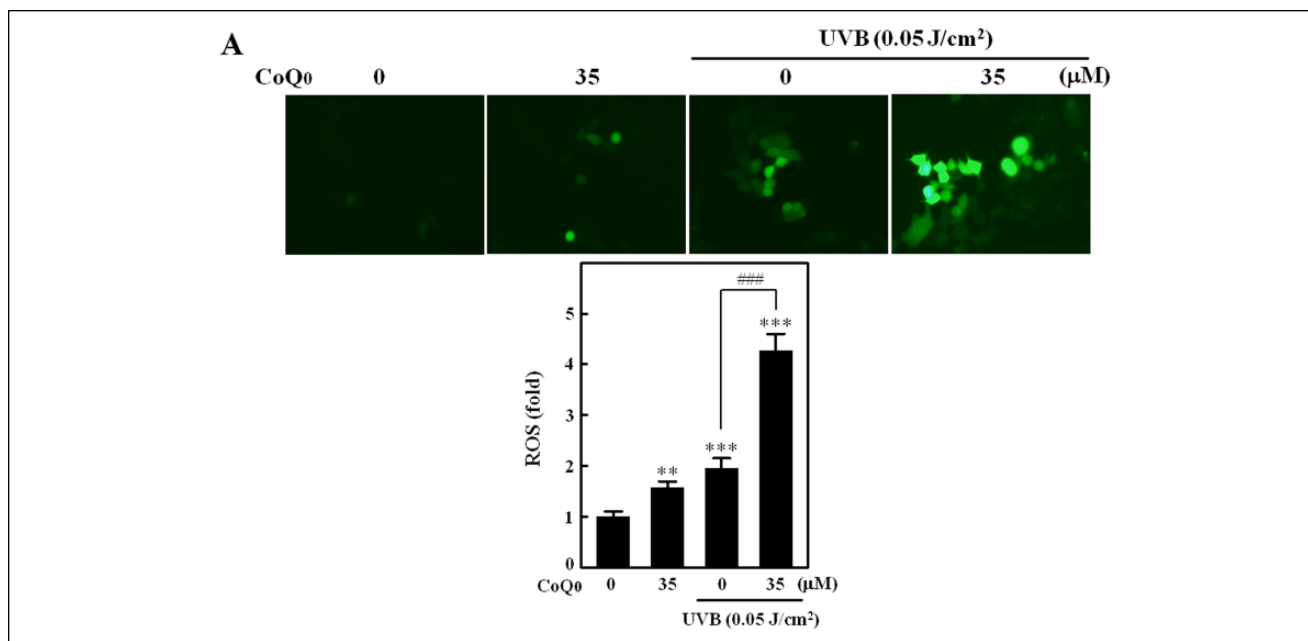
Figure 3. CoQ_0 enhances UVB-induced ROS production in MCF-7 cells. Cells were irradiated with UVB (0.05 J/cm^2) and then treated with CoQ_0 (35 µM) for 20 minutes. (A) The intracellular ROS levels indicated by DCF fluorescence was measured by fluorescence microscopy (200× magnification). (B) Data are presented as fold-change along with fluorescent image. The results are the mean ± SD of 3 assays. *$P < .05$; **$P < .01$; ***$P < .001$ compared with untreated control cells. #$P < .05$; ##$P < .01$; ###$P < .001$ compared with UVB-irradiated cells.

which the efficacy of new therapeutic agents is evaluated in preclinical studies.[36] The results of the present study indicate that CoQ_0 could be an anti–breast cancer agent in vitro. Our findings demonstrated that UVB pretreatment significantly induced apoptosis in MCF-7 cells, which was associated with the excessive production of ROS, DNA fragmentation, followed by a sequence of events, including PARP degradation, Bcl-2/Bax dysregulation, and p53 expression. Taken together, our findings are much more enlightening and revealed that CoQ_0 is effective in the induction of apoptosis through accumulation of ROS in UVB-irradiated MCF-7cells.

Ubiquinone analogs have been shown to exhibit strong pro- or anti-oxidant properties.[37] They generate functional and structural damage through hypergeneration of ROS production.[38] On the contrary, they are also shown to reduce oxidative stress-induced cell death[39] by a mechanism that may involve their antioxidant properties. Ubiquinone analogs display noticeable tissue specificity when compared to other classes of PTP regulators. Studies have confirmed that the production of ROS and the regulation of PTP opening by ubiquinone analogs changes depending on the cell lines (which prevent any extrapolation from results obtained with liver mitochondria). Ubiquinone analogs represent a newly identified family of PTP regulators.[37,40,41] Regardless of their partition coefficient or redox status, ubiquinone analogs are classified in 3 functional groups. According to

whether they favor or inhibit PTP opening or are neutral but compete with active quinones, they are called PTP-inhibitory, PTP-activatory, or PTP-inactive quinones, respectively.

A large number of clinical and preclinical studies exhibit that coenzyme Q (CoQ) or ubiquinone analogs have therapeutic potential effects for inflammation, cancer, and several metabolic disorders.[19,42,43] CoQ is a lipophilic molecule found in the inner side of the mitochondrial membrane of most aerobic organisms from bacteria to mammals. CoQ is composed of a redox active benzoquinone ring conjugated to an isoprenoid chain. The subscript in CoQ molecule refers to the number of isoprene repeat units, which include from CoQ_0 to CoQ_{10}.[42] Various analogs of CoQ have been shown to indicate either pro-oxidant or antioxidant properties. Antioxidant property has been represented by preventing oxidative stress-induced cells death.[21,44] CoQ_0, a redox-active compound without isoprenoid side chain that predominantly accumulates in mitochondria, has been shown to inhibit the activity of complex 1 of the mitochondrial respiratory chain and to prevent opening of the mitochondrial permeability transition pore.[45] We hypothesize that CoQ_0, in the presence of UVB, dramatically changes mitochondrial function, which abundantly increases oxidative stress leading to subsequent mitochondrial permeability transition pore opening and induction of apoptosis in human breast cancer cells.
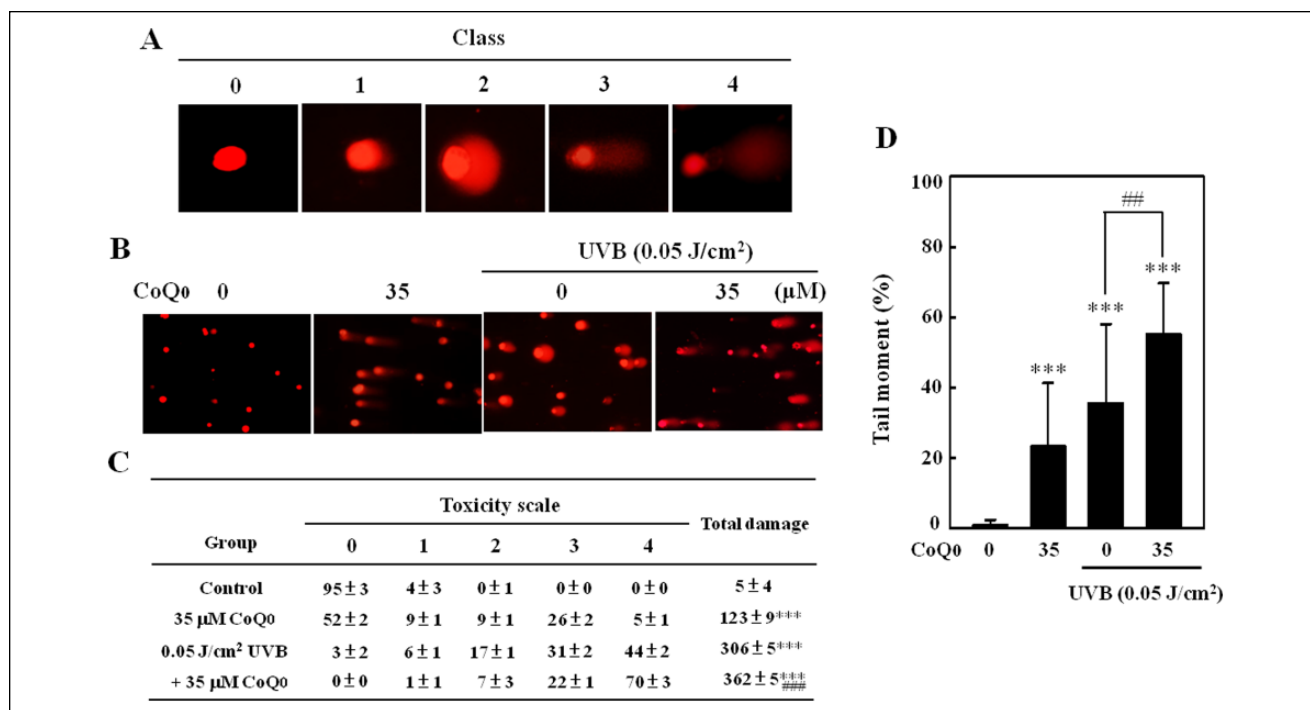
Figure 4. CoQ$_0$ increases UVB-induced DNA damage in MCF-7 cells as assessed by an alkaline comet assay. (A) The cellular DNA was stained with DAPI and photographed using a fluorescence photomicroscope. The comet-like DNA formations were categorized into 5 classes (0, 1, 2, 3, or 4) representing increasing DNA damage shown as a "tail." Each comet was assigned a value according to its class. (B and C) Cells were irradiated with UVB (0.05 J/cm^2) and then treated with CoQ$_0$ (35 μM) for 72 hours. The overall score for 100 cells ranged from 0 (100% of comets in class 0) to 400 (100% of comets in class 4). The observation and the analysis of the results were always performed by the same experienced person. The analysis was blinded, with the observer having no knowledge of the slide identity. (D) The tail moment was quantified as described in Materials and Methods. The results are the mean ± SD of 3 assays. *$P < .05$; **$P < .01$; ***$P < .001$ compared with untreated control cells. #$P < .05$; ##$P < .01$; ###$P < .001$ compared with UVB-irradiated cells.

UVB is also a well-known genotoxic agent that can stimulate oxidative stress through the formation of ROS, such as superoxide anion radical, hydroxyl radical, and hydrogen peroxide, which leads to cell damage and DNA lesions.[46] UVB-induced apoptosis involves a highly complex mechanism. UVB activates death receptors, such as Fas, which eventually leads to the induction of apoptosis. However, nuclear DNA damage is considered as an important factor in UVB-induced apoptosis.[47] Furthermore, exposure to UVB alters the protein function by directly interacting with aromatic amino acids in proteins. However, it has been reported that in the presence of indole-3-carbinol, UVB induced apoptosis in G361 cells through DNA fragmentation, caspase-3 activation, and PARP cleavage.[48] UVB indirectly damages biomolecules through production of ROS and reactive nitrogen species by oxidizing lipids, proteins, and DNA. In addition, UVB generates ROS, which also play major roles in the execution of apoptotic cell death in an independent way.[49] In the present study, our results demonstrated that the combination of UVB and CoQ$_0$ caused significant cell death compared to UVB and CoQ$_0$ alone.

We speculate that CoQ$_0$ may be transformed to potent cytotoxic radicals under UVB irradiation, which subsequently leads to cell death.

Apoptosis-inducing agents are being examined as tools for the management of cancer treatment. Apoptosis is characterized by a number of well-defined features, including cellular morphological changes, internucleosomal DNA cleavage, chromatin condensation, and the activation of the caspase family.[50] Apoptosis activation occurs mainly through 2 well-characterized pathways in mammalian cells, death-receptor-dependent (extrinsic) and mitochondrial-dependent (intrinsic) apoptosis signaling cascades.[51] Recently, we also determined that the induction of apoptosis by AC treatment is mediated by the activation of both mitochondrial and death-receptor pathways.[52] In the present study, comet assays demonstrated that pretreatment of MCF-7 cells with UVB markedly induced apoptotic cell death associated with internucleosomal DNA fragmentation. Furthermore, UVB pretreatment significantly increased the sub-G1 accumulation in MCF-7 cells compared to control group. Cells undergoing apoptosis were
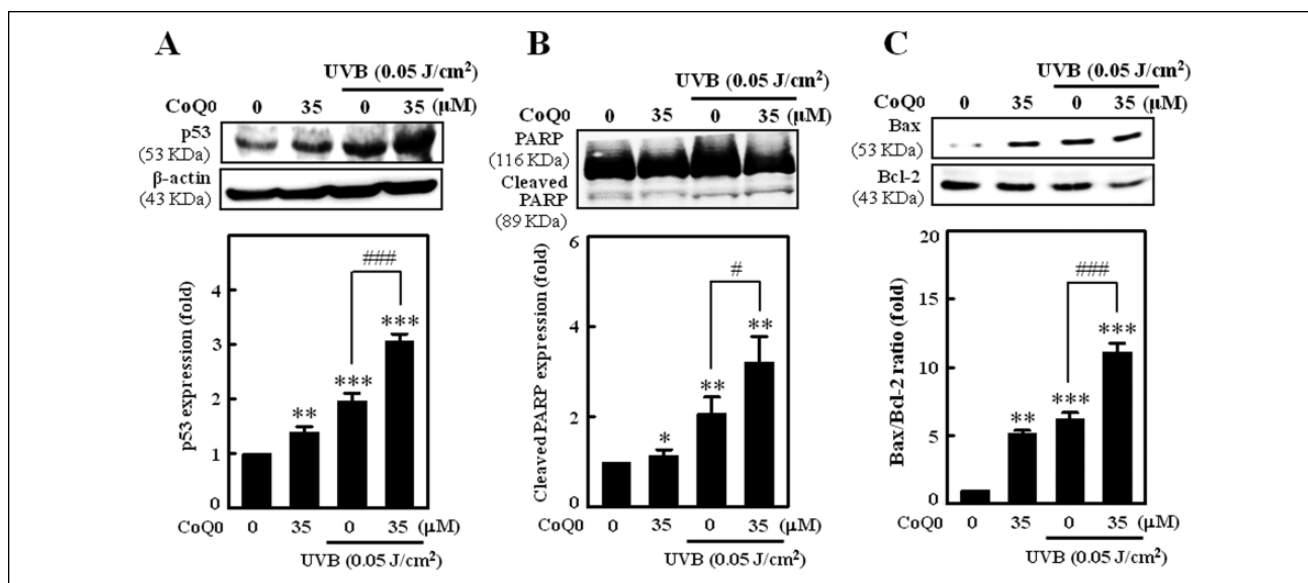
Figure 5. Effect of CoQ_0 on apoptotic proteins in UVB-irradiated MCF-7 cells. Cells were irradiated with UVB (0.05 J/cm^2) and then treated with CoQ_0 (35 μM) for 72 hours. (A-C) The protein levels of p53, PARP, Bax, and Bcl-2 were determined by Western blot. Relative changes in the Bcl-2 and Bax protein bands were measured by commercially available quantitative software (AlphaEase, Genetic Technology Inc, Miami, FL), with the control representing 1.0-fold. The results are the mean ± SD of 3 assays. *P < .05; **P < .01; ***P < .001 compared with untreated control cells. #P < .05; ##P < .01; ###P < .001 compared with UVB-irradiated cells.

found to be associated with elevated levels of cytochrome c in the cytosol with a corresponding decrease in the mitochondria. Cytosolic cytochrome c activates downstream effector caspases (including caspase-3), triggering apoptosis.[53] PARP, a nuclear protein, was shown to be required for apoptosis to proceed in various cell lines. Activated caspase-3 cleaves PARP (116 kDa), generating 89 kDa inactive fragment and cause apoptosis.[54] Our results demonstrated that the combination of CoQ_0 and UVB treatment significantly reduced the pro-form of PARP, which demonstrated that CoQ_0 could act as a chemopreventive agent with respect to inhibition of the growth of MCF-7 cells through the induction of apoptosis.

The excessive generation of ROS can induce oxidative cellular stress, cell cycle arrest, loss of cell function, damage DNA, and apoptosis in cancer cells.[55] Moreover, ROS play a pivotal role in apoptosis by regulating the activity of certain enzymes involved in the cell death pathway.[56,57] ROS triggered the opening of the mitochondrial permeability transition pore, depolarization of the mitochondrial membrane, and then the release of mitochondrial pro-apoptotic factors in the process of mitochondria-mediated apoptosis.[54] In the present study, CoQ_0 and UVB pretreatment remarkably increased the production of intracellular ROS in MCF-7 cells, suggesting that CoQ_0 and UVB might cause a synergistic effect that leads to increase in the intracellular ROS generation. Furthermore, increase in ROS level may act as upstream signaling molecules to induce mitochondria-mediated cell apoptosis in MCF cells.

The p53 tumor suppressor gene plays a critical role in controlling the cell cycle, genome integrity, and apoptosis in response to ionizing radiation, UV light, or certain genotoxic chemicals.[58] p53 activates the expression of target genes when it binds to regulatory DNA sequences, which is important for the inhibition of tumor formation as well as for mediating the cellular responses to many standard DNA damage-inducing cancer therapies by cycle inhibition and induction of apoptosis.[59] Previous studies suggest that p53-regulated apoptosis proceeds primarily through the intrinsic apoptotic program.[60] Accumulating evidence suggest that several natural chemopreventive and chemotherapeutic agents induce apoptosis by activating p53 and its target genes.[61] Research supports that p53 could be activated in response to ROS-dependent nuclear damage and could stimulate the induction of apoptosis.[62] Hence, in order to examine whether the ability of CoQ_0 to induce apoptosis is due to the effect of p53, changes in the protein expression of p53 were examined by western blot. We found that CoQ_0 treatment significantly increased the p53 expression in MCF-7 cells. In addition, UVB pretreatment dramatically increased the p53 level, which clearly indicates that in response to UVB radiation, however, CoQ_0 enhances UVB-induced apoptosis in MCF-7 cells through a p53-dependent signaling pathway.

In mammalian cells, Bcl-2 gene family contains a number of anti-apoptotic proteins, such as Bcl-xL and Bcl-2, which are thought to be involved in resistance to conventional cancer treatment, while the pro-apoptotic proteins

from the same gene family, including Bax, Bak, and Bad, may induce apoptotic cell death.[63] A hallmark of DNA damage-triggered apoptosis is reduced Bcl-2 expression and increased Bax expression. Hence, apoptosis largely depends on the balance between pro-apoptotic and anti-apoptotic protein levels. Activation of the p53 signaling pathway has been shown to play a pivotal role in apoptosis by altering the Bcl-2/Bax ratio.[64] Similarly, the present study indicates a significant inhibition of the anti-apoptotic protein Bcl-2 and a concomitant increase in the expression of the Bax, and p53 proteins by CoQ_0 in UVB-irradiated MCF-7 cells. Taken together, our findings showed that UVB pretreatment enhanced its apoptotic effect possibly by upregulating p53, Bax expression, and downregulating Bcl-2 expression.

Conclusion

In conclusion, CoQ_0 exhibits an antiproliferative effect by induction of apoptosis that is associated with p53 activation, PARP degradation, and alteration of Bcl-2 and Bax in MCF-7 cells. Furthermore, CoQ_0 treatment significantly enhanced UVB-induced apoptosis in MCF-7 cells. As apoptosis has become a new therapeutic target in cancer research, these results confirm that CoQ_0 can be used as a chemotherapeutic or chemopreventive agent that enhances UVB-induced apoptosis in human breast cancer cells. In addition, further investigation of its activity, in vivo, is necessary to elaborate and exploit this nascent promise.

Authors' Note

Authors Hui-Min Wang and Hsin-Ling Yang contributed equally to this study.

Declaration of Conflicting Interests

Funding

References

1. Ho ML, Hsiao YH, Su SY, Chou MC, Liaw YP. Mortality of breast cancer in Taiwan, 1971-2010: temporal changes and an age-period-cohort analysis. *J Obstet Gynaecol*. 2015;35:60-63.

2. Cleator S, Heller W, Coombes RC. Triple-negative breast cancer: therapeutic options. *Lancet Oncol*. 2007;8:235-244.

3. Berry DA, Cronin KA, Plevritis SK, et al. Effect of screening and adjuvant therapy on mortality from breast cancer. *N Engl J Med*. 2005;353:1784-1792.

4. Benson JD, Chen YN, Cornell-Kennon SA, et al. Validating cancer drug targets. *Nature*. 2006;441:451-456.

5. Kim J, Jayaprakasha GK, Patil BS. Obacunone exhibits anti-proliferative and anti-aromatase activity in vitro by inhibiting the p38 MAPK signaling pathway in MCF-7 human breast adenocarcinoma cells. *Biochimie*. 2014;105:36-44.

6. Cha HJ, Kim OY, Lee GT, et al. Identification of ultraviolet B radiation-induced microRNAs in normal human dermal papilla cells. *Mol Med Rep*. 2014;10:1663-1670.

7. Lee CH, Wu SB, Hong CH, Yu HS, Wei YH. Molecular mechanisms of UV-induced apoptosis and its effects on skin residential cells: the implication in UV-Based phototherapy. *Int J Mol Sci*. 2013;14:6414-6435.

8. Kulms D, Schwarz T. Molecular mechanisms of UV-induced apoptosis. *Photodermatol Photoimmunol Photomed*. 2000;16:195-201.

9. Silvan JM, Reguero M, de Pascual-Teresa S. A protective effect of anthocyanins and xanthophylls on UVB-induced damage in retinal pigment epithelial cells. *Food Funct*. 2016;7:1067-1076.

10. Shokrollahi Barough M, Hasanzadeh H, Barati M, Pak F, Kokhaei P, Rezaei-Tavirani M. Apoptosis/necrosis induction by ultraviolet, in ER positive and ER negative breast cancer cell lines. *Iran J Cancer Prev*. 2015;8:e4193.

11. Geethangili M, Tzeng YM. Review of pharmacological effects of *Antrodia camphorata* and its bioactive compounds. *Evid Based Complement Alternat Med*. 2011;2011:212641.

12. Ao ZH, Xu ZH, Lu ZM, Xu HY, Zhang XM, Dou WF. Niuchangchih (*Antrodia camphorata*) and its potential in treating liver diseases. *J Ethnopharmacol*. 2009;121:194-212.

13. Yang HL, Senthik Kumar KJ, Hseu YC. Multiple molecular targets of *Antrodia camphorata*: a suitable candidate for breast cancer chemoprevention. In: Aft R, ed. *Targeting New Pathways and Cell Death in Breast Cancer*. Rijeka, Croatia: InTech; 2012:157-179.

14. Lee CC, Yang HL, Way TD, et al. Inhibition of cell growth and induction of apoptosis by *Antrodia camphorata* in HER-2/neu-overexpressing breast cancer cells through the induction of ROS, depletion of HER-2/neu, and disruption of the PI3K/Akt signaling pathway. *Evid Based Complement Alternat Med*. 2012;2012:702857.

15. Hseu YC, Huang HC, Hsiang CY. *Antrodia camphorata* suppresses lipopolysaccharide-induced nuclear factor-kappaB activation in transgenic mice evaluated by bioluminescence imaging. *Food Chem Toxicol*. 2010;48:2319-2325.

16. Yang HL, Korivi M, Lin MW, Chen SC, Chou CW, Hseu YC. Anti-angiogenic properties of coenzyme Q0 through downregulation of MMP-9/NF-kappaB and upregulation of HO-1 signaling in TNF-alpha-activated human endothelial cells. *Biochem Pharmacol*. 2015;98:144-156.

17. Yang HL, Lin MW, Korivi M, et al. Coenzyme Q0 regulates NFkappaB/AP-1 activation and enhances Nrf2 stabilization in attenuation of LPS-induced inflammation and redox imbalance: evidence from in vitro and in vivo studies. *Biochim Biophys Acta*. 2016;1859:246-261.

18. Chung CH, Yeh SC, Chen CJ, Lee KT. Coenzyme Q0 from *Antrodia cinnamomea* in submerged cultures induces reactive oxygen species-mediated apoptosis in A549 human lung cancer cells. *Evid Based Complement Alternat Med*. 2014;2014:246748.

19. Somers-Edgar TJ, Rosengren RJ. Coenzyme Q0 induces apoptosis and modulates the cell cycle in estrogen receptor negative breast cancer cells. *Anticancer Drugs*. 2009;20: 33-40.

20. MacDonald MJ. Stimulation of insulin release from pancreatic islets by quinones. *Biosci Rep*. 1991;11:165-170.

21. Chen H, Tappel AL. Protection of vitamin E, selenium, trolox C, ascorbic acid palmitate, acetylcysteine, coenzyme Q0, coenzyme Q10, beta-carotene, canthaxanthin, and (+)-catechin against oxidative damage to rat blood and tissues in vivo. *Free Radic Biol Med*. 1995;18:949-953.

22. Knudsen CA, Tappel AL, North JA. Multiple antioxidants protect against heme protein and lipid oxidation in kidney tissue. *Free Radic Biol Med*. 1996;20:165-173.

23. Eggler AL, Gay KA, Mesecar AD. Molecular mechanisms of natural products in chemoprevention: induction of cytoprotective enzymes by Nrf2. *Mol Nutr Food Res*. 2008;52(suppl 1):S84-S94.

24. Thiyagarajan V, Lin SH, Chia YC, Weng CF. A novel inhibitor, 16-hydroxy-cleroda-3,13-dien-16,15-olide, blocks the autophosphorylation site of focal adhesion kinase (Y397) by molecular docking. *Biochim Biophys Acta*. 2013;1830: 4091-4101.

25. Tan W, Lu J, Huang M, et al. Anti-cancer natural products isolated from Chinese medicinal herbs. *Chin Med*. 2011;6(1):27.

26. Thiyagarajan V, Tsai MJ, Weng CF. Antroquinonol targets FAK-signaling pathway suppressed cell migration, invasion, and tumor growth of C6 glioma. *PLoS One*. 2015;10:e0141285.

27. Yang HL, Thiyagarajan V, Liao JW, et al. *Toona sinensis* inhibits murine leukemia WEHI-3 cells and promotes immune response in vivo 2016. *Integr Cancer Ther*. 2016;15(2): 1-11.

28. Hseu YC, Thiyagarajan V, Tsou HT, et al. In vitro and in vivo anti-tumor activity of CoQ0 against melanoma cells: inhibition of metastasis and induction of cell-cycle arrest and apoptosis through modulation of Wnt/beta-catenin signaling pathways. *Oncotarget*. 2016;7:22409-22426.

29. John EM, Schwartz GG, Dreon DM, Koo J. Vitamin D and breast cancer risk: the NHANES I Epidemiologic Follow-Up Study, 1971-1975 to 1992. National Health and Nutrition Examination Survey. *Cancer Epidemiol Biomarkers Prev*. 1999;8:399-406.

30. Dhawan A, Bajpayee M, Parmar D. Comet assay: a reliable tool for the assessment of DNA damage in different models. *Cell Biol Toxicol*. 2009;25:5-32.

31. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*. 1988;175:184-191.

32. Nousis L, Doulias PT, Aligiannis N, et al. DNA protecting and genotoxic effects of olive oil related components in cells exposed to hydrogen peroxide. *Free Radic Res*. 2005;39: 787-795.

33. Wang Z, Sun Y. Targeting p53 for novel anticancer therapy. *Transl Oncol*. 2010;3:1-12.

34. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev*. 1999;13:1899-1911.

35. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*. 2010;127:2893-2917.

36. Buque A, Muhialdin J, Munoz A, et al. Molecular mechanism implicated in Pemetrexed-induced apoptosis in human melanoma cells. *Mol Cancer*. 2012;11:25.

37. Devun F, Walter L, Belliere J, Cottet-Rousselle C, Leverve X, Fontaine E. Ubiquinone analogs: a mitochondrial permeability transition pore-dependent pathway to selective cell death. *PLoS One*. 2010;5:e11792.

38. Bellomo G, Thor H, Orrenius S. Modulation of cellular glutathione and protein thiol status during quinone metabolism. *Methods Enzymol*. 1990;186:627-635.

39. Armstrong JS, Whiteman M, Rose P, Jones DP. The coenzyme Q10 analog decylubiquinone inhibits the redox-activated mitochondrial permeability transition: role of mitochondrial complex III. *J Biol Chem*. 2003;278:49079-49084.

40. Fontaine E, Bernardi P. Progress on the mitochondrial permeability transition pore: regulation by complex I and ubiquinone analogs. *J Bioenerg Biomembr*. 1999;31:335-345.

41. Walter L, Miyoshi H, Leverve X, Bernard P, Fontaine E. Regulation of the mitochondrial permeability transition pore by ubiquinone analogs. A progress report. *Free Radic Res*. 2002;36:405-412.

42. Quinzii CM, Hirano M. Coenzyme Q and mitochondrial disease. *Dev Disabil Res Rev*. 2010;16:183-188.

43. Lee BJ, Tseng YF, Yen CH, Lin PT. Effects of coenzyme Q10 supplementation (300 mg/day) on antioxidation and anti-inflammation in coronary artery disease patients during statins therapy: a randomized, placebo-controlled trial. *Nutr J*. 2013;12:142.

44. Kelso G, Porteous C, Hughes G, et al. Prevention of mitochondrial oxidative damage using targeted antioxidants. *Ann NY Acad Sci*. 2002;959:263-274.

45. Fontaine E, Ichas F, Bernardi P. A ubiquinone-binding site regulates the mitochondrial permeability transition pore. *J Biol Chem*. 1998;273:25734-25740.

46. Nishigori C, Hattori Y, Toyokuni S. Role of reactive oxygen species in skin carcinogenesis. *Antioxid Redox Signal*. 2004;6:561-570.

47. Zeise E, Weichenthal M, Schwarz T, Kulms D. Resistance of human melanoma cells against the death ligand TRAIL is reversed by ultraviolet-B radiation via downregulation of FLIP. *J Invest Dermatol*. 2004;123:746-754.

48. Kim DS, Jeong YM, Moon SI, et al. Indole-3-carbinol enhances ultraviolet B-induced apoptosis by sensitizing human melanoma cells. *Cell Mol Life Sci*. 2006;63: 2661-2668.

49. Kulms D, Zeise E, Poppelmann B, Schwarz T. DNA damage, death receptor activation and reactive oxygen species contribute to ultraviolet radiation-induced apoptosis in an essential and independent way. *Oncogene*. 2002;21:5844-5851.

50. Lin E, Lin WH, Wang SY, et al. Flavokawain B inhibits growth of human squamous carcinoma cells: involvement of apoptosis and cell cycle dysregulation in vitro and in vivo. *J Nutr Biochem*. 2012;23:368-378.

51. Chowdhury I, Tharakan B, Bhat GK. Current concepts in apoptosis: the physiological suicide program revisited. *Cell Mol Biol Lett*. 2006;11:506-525.

52. Hseu YC, Tsou HT, Kumar KJ, Lin KY, Chang HW, Yang HL. The antitumor activity of *Antrodia camphorata* in melanoma cells: modulation of Wnt/beta-catenin signaling pathways. *Evid Based Complement Alternat Med*. 2012;2012:197309.

53. Sanchez-Alcazar JA, Khodjakov A, Schneider E. Anticancer drugs induce increased mitochondrial cytochrome c expression that precedes cell death. *Cancer Res*. 2001;61:1038-1044.

54. Wang X. The expanding role of mitochondria in apoptosis. *Genes Dev*. 2001;15:2922-2933.

55. Hua P, Sun M, Zhang G, et al. Cepharanthine induces apoptosis through reactive oxygen species and mitochondrial dysfunction in human non-small-cell lung cancer cells. *Biochem Biophys Res Commun*. 2015;460:136-142.

56. Garcia-Ruiz C, Colell A, Mari M, Morales A, Fernandez-Checa JC. Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. *J Biol Chem*. 1997;272:11369-11377.

57. Chen Y, Zhang H, Zhou HJ, Ji W, Min W. Mitochondrial redox signaling and tumor progression. *Cancers (Basel)*. 2016;8(4). doi:10.3390/cancers8040040.

58. Bode AM, Dong Z. Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer*. 2004;4:793-805.

59. Lu C, El-Deiry WS. Targeting p53 for enhanced radio- and chemo-sensitivity. *Apoptosis*. 2009;14:597-606.

60. Soengas MS, Alarcon RM, Yoshida H, et al. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science*. 1999;284:156-159.

61. Millimouno FM, Dong J, Yang L, Li J, Li X. Targeting apoptosis pathways in cancer and perspectives with natural compounds from mother nature. *Cancer Prev Res (Phila)*. 2014;7:1081-1107.

62. Vousden KH, Lane DP. p53 in health and disease. *Nat Rev Mol Cell Biol*. 2007;8:275-283.

63. Bargou RC, Wagener C, Bommert K, et al. Overexpression of the death-promoting gene bax-alpha which is downregulated in breast cancer restores sensitivity to different apoptotic stimuli and reduces tumor growth in SCID mice. *J Clin Invest*. 1996;97:2651-2659.

64. Choudhuri T, Pal S, Agarwal ML, Das T, Sa G. Curcumin induces apoptosis in human breast cancer cells through p53-dependent Bax induction. *FEBS Lett*. 2002;512:334-340.