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Effects of quercetin on ionizing radiation-induced cellular responses in HepG2 cells

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ABSTRACT

Background: Quercetin has been reported to modulate cell proliferation and apoptosis. The present study aimed at identifying whether treatment of ionizing radiation (IR) combined with quercetin induces apoptosis in HepG2 cells. ***Materials and Methods***: HepG2 cells were plated at an appropriate density according to each experimental scale and irradiated with 1, 5 and 10 Gy gamma-rays from a ^{60}Co source at room temperature. Cell viability, SOD and CAT were assessed by using commercial assay kits. Western blot analyses were done on apoptosis related proteins. The cells were treated with various concentrations of quercetin alone or in combination with IR. ***Results:*** The cell viability was decreased in a concentration-dependent manner 24 h after treatment of quercetin. It was significantly lowered after the combined treatment of quercetin with IR than that of the cells treated with quercetin alone. Moreover, quercetin increased the expression of p53 levels in a dose-dependent manner. Combined treatment of quercetin with IR significantly increased the levels of pro-apoptotic proteins, cleaved caspase-3 and caspase -7, and Bax. Cell cycle analyses indicated a drastic increase in the Sub G1 population after quercetin treatment combined with IR. The activity of caspase-3 increased coincidently with apoptosis. The combined treatment of quercetin with IR decreased catalase and superoxide dismutase activities, as well. ***Conclusion:*** Quercetin made the radio-resistant HepG2 cells undergo apoptosis by activating p53. These results suggest that the combined treatment of quercetin with IR may provide an effective therapeutic strategy to improve the radiotherapy efficacy.

Keywords: *Quercetin, apoptosis, autophagy, human hepatocellular carcinoma cell, radiosensitizer.*

INTRODUCTION

Exposure of cells to IR can result in lethal damage with the induced double-strand breaks of DNA if left unrepaired [1]. The exposed cells show various effects such as growth arrest, senescence, necrosis or mitotic catastrophe, apoptosis and autophagy [2, 3].

IR-induced apoptosis, alias type I programmed cell death, has been intensively studied over the past decade [4]. Stereotypical morphological changes such as cell shrinkage, membrane blebbing and nuclear fragmentation are characteristics of apoptosis. Chromatin condensation is, in particular, activated by caspases and regulated by the Bcl-2 family members [5, 6].

It has been reported that irradiation initiates a type II programmed cell death (autophagy) [7]. Autophagy does not refer to a death process, but a self-cannibalization process through a lysosomal degradation pathway. It is one of the major regulated mechanisms of cells for degrading long-lived proteins, which can be

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triggered by physiologic stress such as nutrient deprivation, or by another pathway [8,9,10]. Autophagy can be activated to prevent cancer cells from death. The studies using cell lines defective in apoptosis indicated that autophagy was triggered in response to deprivation of growth factors, nutrient depletion or hypoxia to give rise to survival advantage for tumor cells [11]. Accordingly, many clinical trials vigorously attempt to elucidate the specific role of autophagy in cancer treatments [12].

HepG2, human hepatocellular carcinoma cell, is an excellent experimental model because it is easy to culture, and widely used for biochemical studies where many conditions can be analyzed with minor inter-assay variations [13]. The relative resistance of liver cancer cells to IR originates from autophagic response is the limitation on the available treatment options for this type of cancer. Tseng *et al.* (2011) used HepG2 ($p53^{+/+}$) and Hep3B ($p53^{-/-}$) cells to examine the radio-resistance of liver cancer cells. According to their results, HepG2 cells were radio-resistant through the IR-induced autophagy pathway whereas Hep3B cells underwent apoptotic cell death in response to irradiation [14].

Quercetin is a flavone-3ol-class of flavonoid which ubiquitously presents in fruits and vegetables. It has been reported that quercetin modulates signal transduction pathways related to cell proliferation and apoptosis [15]. Many results from *in-vitro* experiments demonstrate that quercetin inhibits cell proliferation and accordingly induces apoptosis in several types of cancer cells through different signaling pathways [16, 17, 18, 19]. It has been shown that quercetin gives birth to selective apoptosis-inducing activity and antiproliferative effects in cancer cells but not in normal cells. In that regard, quercetin may serve as an anticancer agent or as an adjunct to cancer therapies currently available [20, 21]. Studies on the molecular mechanisms underlying the quercetin's antiproliferative effects have indicated that quercetin treatments triggered numerous cellular events including cell cycle arrest at G1 and/or G2/M stage, p53 activation and induction of caspase-mediated apoptosis in some cancer cells [22].

Accordingly the present study was carried out to evaluate apoptotic effects of quercetin, as an inhibitor of autophagy, on HepG2 cells, and to elucidate the function of quercetin as a drug candidate for a useful strategy to improve the efficacy of radiotherapy, as well.

MATERIALS AND METHODS

Materials

Quercetin (CAS No. 23513-14-6) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and DMEM media from Welgene Inc. (Daegu, Korea). Fetal Bovine Serum (FBS) and Phosphate Buffered Saline (PBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Antibiotic-antimycotic and Trypsin-EDTA were obtained from Gibco (Gaithersburg, MD, USA). EZ-CyTox Cell Viability, Proliferation & Cytotoxicity Assay Kits were obtained from DOGEN (Seoul, Korea). Catalase (CAT) and Superoxide Dismutase (SOD) assay kits were from Cayman Chemical Company (MI, USA). Caspase-3/CPP32 colorimetric assay kit was from BioVision (USA). Poly-L-lysine and propidium iodide were from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were used: p53, p21, Bax, Bcl-2 and β-actin from Santa Cruz (Santa Cruz, CA, USA); LC3 from novus (Littleton, CO, USA); Cleaved caspase-3 and cleaved caspase-7 from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell culture and irradiation

Human hepatocellular carcinoma cell line (HepG2 cells) was obtained from American Type Culture Collection (ATCC, USA). Cells were maintained in DMEM medium supplemented with 10% FBS and antibiotic-antimycotic at appropriate concentration and 5 μg/ml plasmocin (InvivoGen, CA, USA) at 37 °C with 5% CO_2 in a fully humidified atmosphere. Cells were plated at an appropriate density according to each experimental scale and irradiated with 1, 5 and 10 Gy gamma-rays from ^{60}Co source (source strength 42.6 TBq, Korea Atomic Energy

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Research Institute) at room temperature. Radiation doses were chosen based on the dose-response relationship of γ-H2AX foci in the cultured cells which was reported to IAEA [23].

Cell viability analysis

The quercetin's effects on cell viability were assessed by using the EZ-CyTox cell viability assay kits. HepG2 cells were seeded at a density of 5×10^3 cells in each well of the 96 well plates. After incubation for additional 24 h, the cells were treated with quercetin in 10-100 µM concentration for 24 h or 48 h. The Cell Viability Assay Reagent was then added and incubated for 1 h at 37 °C with 5% CO_2. The absorbance was read at 450 nm.

DAPI staining

Nucleic acid staining with DAPI (4', 6'-diamidine-2'-phenylindole dihydrochloride) was done to observe the level of nuclear condensation and fragmentation. The HepG2 cells were cultured on cover slips coated with poly-L-lysine and treated with quercetin. After treatment, the cells were washed with PBS and fixed with 100% methanol at room temperature for 5 min. After washing, DAPI solution was added in a concentration of 5 µg/ml for 15 min at room temperature. After washing, fluorescence images were taken under a fluorescence microscope (BX50, Olympus Corporation, Tokyo, Japan) with SPOT software 5.1 (Diagnostic Instrument, USA).

Western blot analysis

Western blotting was applied to analyze the protein expression levels. After treatment of the HepG2 cells with IR in the presence or absence of quercetin, the cells were washed with Phosphate Buffered Saline (PBS), and then lysed in an ice-cold PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Gyeonggi-do, Korea). The soluble fraction of the cell lysates was isolated by centrifugation at 13,000 x g for 20 min in a microfuge. Supernatants were measured for protein concentration using a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA, USA). The equal amounts of protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and then transferred to membranes, which were blocked for 2 h at room temperature with 5% non-fat dried milk in TBS-T (10 mM Tris-HCl [pH 7.4], 150 mM NaCl and 0.1% Tween-20) and then incubated with primary antibodies overnight at 4 °C. The blots were washed three times for 5 min with TBS-T, then incubated for 2 h with peroxidase-conjugated secondary antibody (1:4,000) at room temperature. The membranes were washed three times for 5 min and developed using Pierce ECL Western Blotting Substrate (Thermo scientific, Rockford, Illinois).

Cell cycle analysis

After quercetin treatment in combination with IR for 48 h, floating and trypsin-detached HepG2 cells were collected by centrifugation. The cells were washed with PBS and then fixed with 70% ice-cold ethanol overnight at -20 °C, and then treated with 100 mg/ml RNase A and 50 mg/ml propidium iodide. To analyze apoptosis, sub G1 (hypodiploid DNA) populations were assayed using a Cytomics FC 500 flow cytometer with Cytometer CXP software (Beckman Coulter, Franklin Lakes, NJ, USA). The results represent the means of triplicate determinations in which a minimum of 10,000 cells were assayed for each determination. Any sub G1 population was counted as apoptotic cells.

Caspase-3 activity assay

Assay for caspase-3 activity was done according to the manufacturer's instruction (Caspase-3/CPP32 colorimetric assay kit, BioVision, USA). The cells were collected using the rubber policeman and the resultant pellets were sonicated for three 2 s bursts in 300 µl of 0.1 M Tris-HCl [pH 7.5] and centrifuged at 13,000 x g for 15 min at 4 °C. 50 µg of proteins (diluted to 50 µl Cell Lysis Buffer) was used for each assay and 50 µl of 2X Reaction Buffer was added to each sample. After adding 5 µl of the 4 mM DEVD-pNA substrate, the samples were incubated at 37 °C for 24 h. The absorbance was measured at 405 nm by using a plate reader.

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Catalase (CAT) activity assay

Assay for CAT activity was performed according to the manufacturer's instruction (Cayman Chemical Company, USA). The decomposition of H_2O_2 in presence of CAT was followed by the decrease in absorbance measured using a spectrophotometer. The cells were collected using the rubber policeman and the resultant pellets were sonicated in 300 µl of 0.1 M Tris-HCl [pH 7.5] and centrifuged at 13,000 x g for 15 min at 4 °C. The supernatants were used for the assay and stored on ice. Protein concentration was determined by the Bradford method [24]. Five µg (diluted to 30 µl of Tris-HCl [pH 7.5]) of the sample, 100 µl of 100 mM potassium phosphate [pH 7.0], and 30 µl of methanol were added in each well of 96 well plates. The reaction was initiated by adding 20 µl of hydrogen peroxide to each well and incubated on a shaker for 20 min at room temperature. To terminate the reaction, 30 µl of potassium hydroxide was added to the each well and then 30 µl of catalase purpald (chromogen) was added as quickly as possible. The plate was incubated for 10 min at room temperature on the shaker. Finally, 10 µl of catalase potassium periodate was added to each well and incubated for 5 min at the room temperature on the shaker. The absorbance was taken at 540 nm by using a plate reader.

Superoxide Dismutase (SOD) activity assay

Assay for SOD activity was performed according to the manufacturer's instruction (Cayman Chemical Company, USA). Briefly, the cells were collected using the rubber policeman and the resultant pellets were sonicated in 300 µl of 0.1M Tris-HCl [pH 7.5] and centrifuged at 13,000 x g for 15 min at 4 °C. The supernatants were collected for assay and stored on ice. Protein concentration was determined by Bradford method [24]. One unit of enzyme activity was defined as the 50% dismutation of superoxide radical. In a 96 well plate, 200 µl tetrazolium salt was added to 5 µg of each sample (diluted to 10 µl of Tris-HCl [pH 7.5]). The reaction was initiated by adding 20 µl of Xanthine oxidase. After incubation of the plate on a shaker for 20 min at room temperature, the absorbance was measured at 450 nm by a plate reader.

Statistical analysis

All the presented data and results were confirmed in three independent experiments. The data are expressed as mean ± standard error of the mean. Statistical comparisons were made by Student's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

IR induced autophagy in HepG2 cells

The viability of the HepG2 cells was evaluated using an EZ-CyTox cell viability assay kit. It was found that cell viability was not affected at the indicated incubation time (figure 1A). An autophagic marker LC3 was identified in the HepG2 (figure 1B). LC3, a useful marker of autophagic membranes, is present in a free cytoplasmic form as LC3-I. LC3-II is produced when it is associated with phosphatidylethanolamine (through an ubiquitin-like conjugation reaction) of the membrane of autophagosome [25]. Autophagosome-incorporated LC3-II protein expression induced by IR increased 24 h after irradiation compared to the control. Significant change in LC3-II was detected following IR treatment in a dose-dependent manner. These results suggest that IR-induced autophagy results in radio-resistance of HepG2 cells under this experimental condition.

Quercetin reduced the viability of HepG2 cells

Quercetin (figure 2A) treatment resulted in a concentration dependent inhibition of cell viability in the HepG2 cells. To investigate morphological changes, The HepG2 cells were treated with 0, 10 and 100 µM of quercetin for 48 h (figure 2B). The results showed cell shrinkage with irregularities in shape and cells became sharpened in a dose-dependent manner, which indicate the cytotoxicity of quercetin in HepG2 cells. The effect of quercetin on the viability of HepG2 cells were evaluated at 24 h and 48 h incubation time in quercetin-containing

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medium at different concentrations (10, 20, 40, 80 and 100 µM). It was revealed that quercetin reduced the HepG2 cell's viability, in a concentration-dependent manner. In particular, a maximum reduction in the cell viability was observed at 48 h post-treatment at a concentration of 100 µM as 31 % (figure 2C).

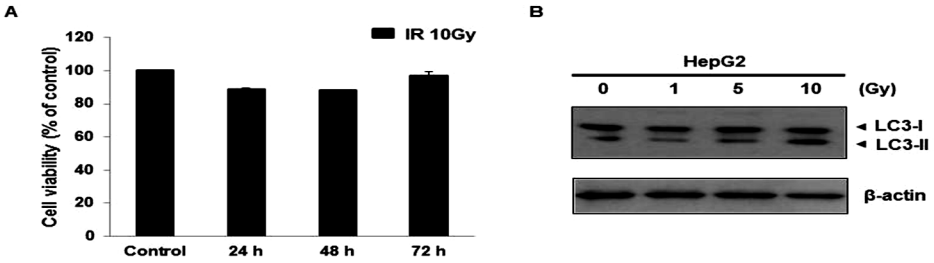


Figure 1. The effect of IR on cell viability and autophagy in HepG2 cells. (A) Time course of cell viability in HepG2 cells after irradiation. HepG2 cells were incubated for an indicated time after irradiation. Cell viability was analyzed using EZ-CyTox cell viability assay kit, as described in "Materials and Methods". Data represents the average results of three independent experiments (mean±SEM). Cell viability was expressed as mean percentage of the control (100%). (B) IR-induced autophagy in HepG2 cells. HepG2 cells were irradiated and incubated for 48 h. The level of LC3 protein was analyzed using western blot analysis, as described in "Materials and Methods". The upper band is LC3-I and the lower band is LC3-II.

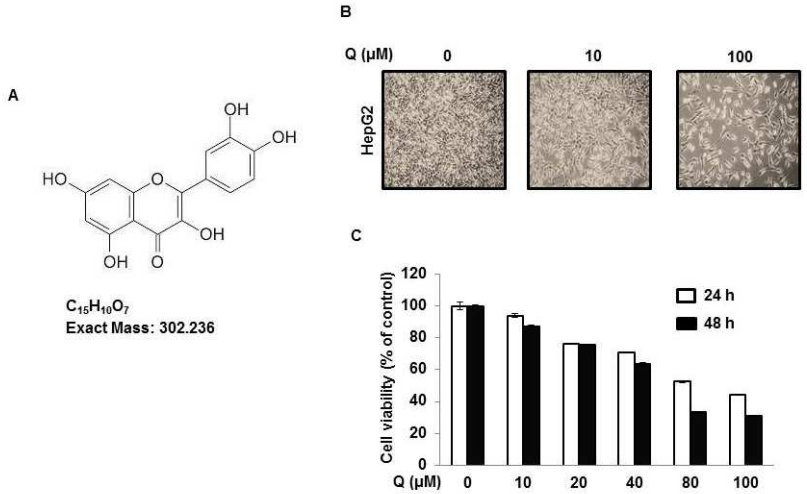


Figure 2. The effect of quercetin on cell viability in HepG2 cells. (A) The chemical structure of quercetin. (B) Cells were incubated with quercetin (0, 10, 20, 40, 80 and 100 µM) for 24 h and 48 h. Cell viability of HepG2 cells after quercetin treatment was determined using an EZ-CyTox cell viability assay kit. Data represents the average results of three independent experiments (mean±SEM). Cell viability was expressed as mean percentage of the control (100%). (C) Morphological changes were observed in HepG2 cells treated with quercetin (0, 10 and 100 µM) for 48 h.

Combined treatment of quercetin and IR reduced the viability of HepG2 cells

To find out whether combined treatment of quercetin and IR sensitizes the HepG2 cells to apoptosis, cell morphology was examined and cell viability assay and DAPI staining were conducted. Morphological change of HepG2 cells were clearly shown after the combined treatment of quercetin and IR. Cell rounding, shrinkage and irregularities in shape were shown, which indicates the increase of apoptosis of the quercetin-treated HepG2 cells in a concentration-dependent manner (figure 3A). Also, co-treatment of quercetin and IR decreased cell viability compared to cells treated with quercetin alone (figure 3B). As shown in figure 3C, nuclear condensation and fragmentation increased in co-treated cells compared to the control cells.

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Quercetin regulated the expressions of pro-apoptotic proteins and increased apoptosis in HepG2 cells

To find out whether the treatment of cells with combined treatment of quercetin and IR regulates pro-apoptotic proteins, the protein expression levels of p53, p21, Bax, Bcl-2, cleaved caspase-3, -7 and LC3 were detected by western blot analysis. It was observed that p53, p21 and representative pro-apoptotic proteins (Bax, cleaved caspase-3 and caspase-7) were increased after combined treatment of quercetin and IR (figure 4A). After combined treatment of quercetin and IR, p53 and its down-stream proteins were increased more than cells treated with each treatment alone. Autophagosome-incorporated LC3-II protein was reduced following quercetin treatment, but the conversion of LC3-I to LC3-II was shown after irradiation with 10 Gy. These results implied IR induced autophagy in HepG2 cells, which resulted in radio-resistance. In order to evaluate whether quercetin and IR co-treatment induce apoptosis, cell cycle analysis using flow cytometry was performed (figure 4B). A drastic increase in Sub G1 population was shown after combined treatments of quercetin and IR. The further examinations were conducted to determine whether this radio-sensitizing effect of quercetin is associated with caspase-3 activation (figure 4C). The activity of caspase-3 was increased in cells exposed to quercetin or IR alone, and 1.4 fold increase was detected after combined treatments of quercetin and IR. These results suggest that quercetin induces apoptosis through the activation caspase-3 activity. Therefore, quercetin can be function as a radio-sensitizer by increasing caspase-3 activity, which resulted in apoptotic cell death in the HepG2 cells.

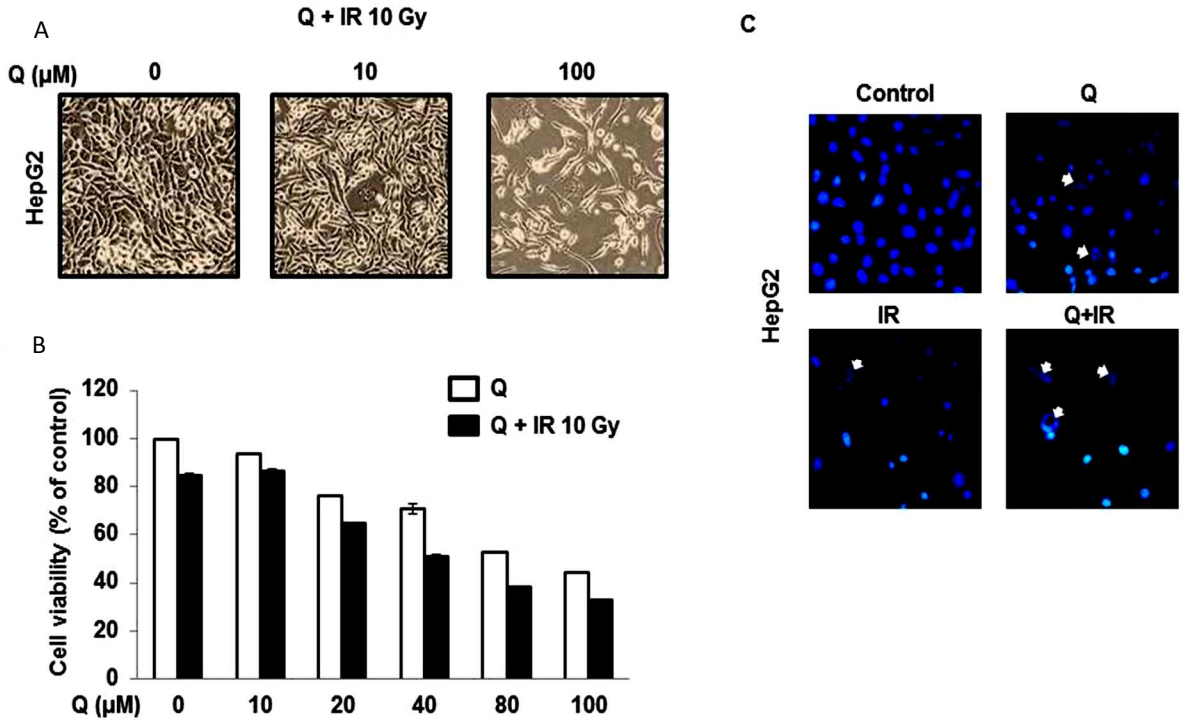


Figure 3. The effect of combined treatment of quercetin and IR on cell viability in HepG2 cells. (A) HepG2 cells were exposed to different concentrations of quercetin with or without 10 Gy irradiation. Cell viability was determined using an EZ-CyTox cell viability assay. Data represents the average results of three independent experiments (maen±SEM).. Cell viability was expressed as mean percentage of the control (100%). (B) Morphological changes of HepG2 cells with 10 Gy irradiation after pre-treatment of quercetin (0, 10 and 100 μM). (C) Cells were incubated with quercetin (0 and 100 μM) for 48 h. The cells were then prepared for DAPI staining as described in "Materials and Methods".

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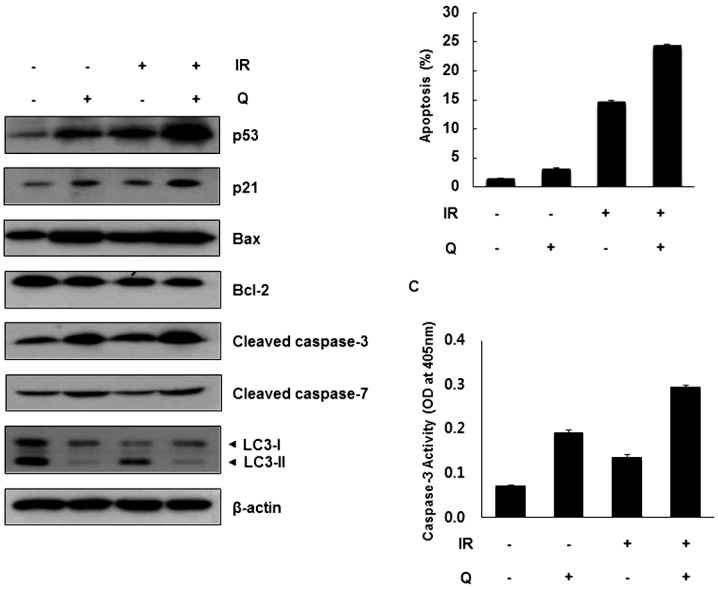


Figure 4. The effect of combined treatment of quercetin and IR on apoptosis in HepG2 cells. (A) HepG2 cells were exposed to quercetin (100 μM) for 48 h with or without irradiation (10 Gy), and the protein expressions of p53, p21, Bax, Bcl-2, cleaved caspase -3, cleaved caspase-7 and LC3 were determined by western blotting. Forty micrograms of cellular extract per lane was separated on 12% SDS-PAGE gel. Equal protein loading and protein transfer were confirmed with anti-β-actin antibody. (B) Cells were exposed to quercetin (100 μM) for 48 h with or without 10 Gy irradiation. The cells were then harvested and prepared for PI staining as described in "Materials and Methods". Sub G1 population was analyzed using flow cytometry and Sub G1 population was counted as apoptotic cells. (C) The effect of the combined treatment of quercetin and IR on the caspase-3 activity. HepG2 cells were irradiated with 10 Gy and treated with 100 μM quercetin for 48 h. Data represents the average results of three independent experiments (mean± SEM).

Quercetin regulated CAT and SOD activity in HepG2 cells

To evaluate the effect of quercetin on the antioxidant enzyme activities of the HepG2 cells, assays for CAT and SOD activity were performed. Treatment of quercetin or IR alone decreased 10% of CAT activity (Fig. 5A). However, CAT activity was more decreased to 35 % after combined treatment of quercetin and IR. SOD activity showed similar pattern of decreased activity after co-treatment of quercetin and IR (figure 5B).

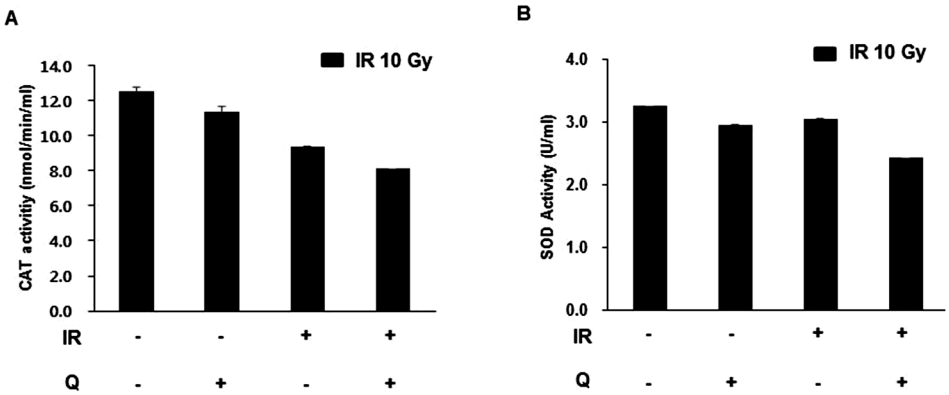


Figure 5. The effect of combined treatment of quercetin and IR on catalase (CAT) and superoxide dismutase (SOD) acitivity. (A) CAT activity was analyzed using CAT assay kit. Changes of CAT levels in HepG2 cells were expressed as nmol/min/ml. (B) SOD activity was analyzed using SOD assay kit. Changes of SOD levels in HepG2 cells were expressed as U/ml. Three independent experiments were performed in triplicates and mean ±SD has been plotted.

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DISCUSSION

It was reported that quercetin inhibited cancer growth and induced apoptosis in animal models [26]. Quercetin treatment was associated with the selective effect of antiproliferation on cancer cells and cell death induction, predominantly through an apoptotic mechanism, in cancer cell lines but not in normal cells [27, 28]. The present study focused on the anticancer effects of quercetin, a candidate of pharmacologically active component found in plants. Previous studies demonstrated the anticancer properties of quercetin, including cell cycle arrest, apoptosis and necrosis in a variety of cancer cell lines such as leukemia [29], breast cancer [30], hepatoma [31], oral cancer [32], and colon cancer [33]. Hepatocellular carcinoma (HCC) is the fifth most frequent neoplasm worldwide (>500,000 death/year) [34]. Practical limitations on radiotherapy to treat HCC lie in the inherent tumor radio-resistance and low radiation tolerance of the surrounding normal liver [35].

The previous studies have focused on autophagy since its paradoxical role in survival and death of cells can play its role in cancer therapy when properly manipulated. In this study, radio-resistant HepG2 cells were used to evaluate the role of autophagy in determining the cell's fate. The cell viability was not greatly affected by IR as radio-resistant cells underwent autophagy (figure 1A). IR-induced autophagy was confirmed by the result of western blot analysis for LC3, the marker of autophagy, shown in figure 1B. Similar findings were reported recently by Apel *et al.* [36] and Tseng *et al.* [14], which showed that radiation-induced autophagy contributed to the radio-resistance of carcinoma cells. Tseng's study suggests that autophagy functions as a self-protective mechanism, and its inhibition may be a promising strategy for tailored cancer treatment. Also, the p53 plays an important role in radio-resistance in HepG2 cells through the induction of autophagy. Thus, the present study makes it possible to demonstrate the applicability of quercetin as a radio-sensitizer in HepG2 cells.

In this study, the cytotoxic effect by the treatment of quercetin combined with IR on the HepG2 cells was assessed by the MTT assay. As a traditional method for discovering anticancer drugs, the MTT assay can be used to determine the cytotoxic effect and cell viability of cell lines [37]. While treatment with quercetin alone up to 100 μM reduced cell viability at 24 h or 48 h post-treatment (figure 2C), the combined treatment of quercetin and IR reduced cell viability more than that of the cells treated with quercetin alone (figure 3B). Morphological changes were observed in the HepG2 cells treated with quercetin or simultaneously treated with quercetin and IR for 48 h. The cells treated with 100 μM quercetin alone and cells co-treated with 100 μM quercetin and IR showed cell shrinkage and irregularities in shape (figure 2B and 3A). In the result of DAPI staining, nuclear condensation and fragmentation increased in the co-treated cells compared to those of the control cells (figure 3C).

Apoptosis is a very complex process which is regulated by the interplay between anti- and pro-apoptotic pathways with alterations in the balance between different signaling processes. Exposure to cellular stresses can activate the p53 tumor suppressor to induce cell growth arrest or apoptosis [38]. The p53 has a major role in cellular response to DNA damaging factors [39]. Initialization of DNA damage evolves the up-regulation of p53, which accelerates apoptosis [40]. The expression of some apoptotic proteins assessed by western blotting indicated a significant increase in the expression of p53 in the HepG2 cells simultaneously treated with quercetin and IR. Also, the combined treatment reduced the expression level of Bcl-2, increased the expression level of Bax, activated caspase-3 and caspase-7. These data suggest that quercetin regulated the expression level of p53 and ultimately led to apoptotic cell death (figure 4A). Investigations on autophagy related genes (ATG) show that ATG5, one of ATG family, functions as a switch turning the process of cell death from autophagy to apoptosis [41, 42]. As shown in our result (figure 4A), quercetin treatment, irrespectively of combination with IR, increased pro-apoptotic proteins while IR

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treatment alone resulted in conversion of LC3-I to LC3-II, indicating the autophagy process. The experimental results of this study are in a good agreement with another report which quercetin may contribute to lymphoma prevention by downregulating PI3K-AKT1-p53 pathway as well as by glycolytic metabolism [43]. In this regard, it is possible for quercetin to give rise to certain effects on autophagy related genes' switching function.

As shown in figure 4B an increase in Sub G1 population was observed after the combined treatment of quercetin with IR. Increased p53 by the combined treatment facilitated apoptotic cell death. Apoptotic cell death is activated by a family of caspases which lead to death of the cell [44]. Among them, caspase-3 activity has been detected in apoptosis induced by a variety of apoptotic signals, including IR [45]. It is absolutely crucial for apoptosis induction, as this enzyme is not only activated downstreams of death pathway. It is also responsible for the cleavage of the majority of substrates [46]. Treatment with quercetin combined with IR increased caspase-3 activity coincidently with the increase of apoptotic Sub G1 population (figure 4C).

Enzyme activities of CAT and SOD were decreased after the combined treatment of quercetin and IR (figures 5A and 5B). Recent studies indicate that he p53 affects ROS levels by regulating enzyme activities [18, 47, 48, 49]. Moreover, apoptosis triggered by p53 has been reported to be dependent on the release of apoptotic factors resulting from mitochondrial damage, and on an increase of ROS, as well [50]. The results in this study assume that the increased p53 levels suppress antioxidant enzyme activity, and accordingly lead to cell death by increased ROS levels.

Previous studies have identified the effect of separate treatment of quercetin or IR on tumor cells. There has been no attempt to observe the responses induced by the combined treatment of quercetin with IR. In this study, the radio-resistant HepG2 cells were used to find out the effect of quercetin, as a possible radio-sensitizing agent. Quercetin made the radio-resistant HepG2 cells undergo apoptosis by activating p53. Thus, increased p53 and pro-apoptotic proteins reduced antioxidant enzyme activity to promote apoptosis. These results suggest that the combined treatment of quercetin with IR may provide an effective therapeutic strategy for the treatment of HCC, which are often resistant to radio-therapy.

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Conflicts of interest*: *Declared none.

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