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Induction of cell apoptosis in 3T3-L1 pre-adipocytes by flavonoids is associated with their antioxidant activity

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Obesity is biologically characterized at the cellular level by an increase in the number and size of adipocytes differentiated from fibroblastic pre-adipocytes in adipose tissue. In this study, we focused on the relationship between the influence of flavonoids on cell population growth and their antioxidant activity. The results showed that the inhibition of flavonoids (naringenin, rutin, hesperidin, resveratrol, naringin and quercetin) on 3T3-L1 pre-adipocytes was 28.3, 8.1, 11.1, 33.2, 5.6 and 71.5%, respectively. In oxygen radical absorbance capacity (ORAC) assay, quercetin had the highest ORAC$_{ROO}$ value among the six flavonoids tested. Apoptosis assays showed that quercetin increased apoptotic cells in time- and dose-dependent manner. Treatment of cells with quercetin decreased the mitochondrial membrane potential in the courses of time and dose. The cell apoptosis/necrosis assay showed that quercetin increased the number of apoptotic cells, but not necrotic cells. Quercetin treatment of cells caused a significant time- and dose-dependent increase in the caspase-3 activity. Western analysis indicated that treatment of quercetin markedly down-regulated PARP and Bcl-2 proteins, and activated caspase-3, Bax, and Bak proteins. These results indicate that quercetin efficiently inhibits cell population growth and induction of apoptosis in 3T3-L1 pre-adipocytes.

Keywords: Antioxidant / Apoptosis / Flavonoids / Pre-adipocytes / Quercetin

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1 Introduction

Obesity is one of the main public health problems in developed countries. It is defined as an abnormal increase in body fat, though not necessarily in body weight. However, adipocytes are the primary site of energy storage and accumulate triacylglycerol during nutritional excess. It has been reported that adipocyte dysfunction plays an important role in the development of obesity and insulin resistance [1]. Therefore, it is considered a risk factor associated with the genesis or development of various diseases, including coronary heart disease, hypertension, type 2 diabetes mellitus, cancer, respiratory complications, and osteoarthritis [2]. Recent reports have outlined the mechanisms of proposed anti-obesity including decreased energy and increased energy expenditure, decreased pre-adipocyte differentiation and proliferation, decreased lipogenesis and increased lipolysis, and fat oxidation [3].

Many naturally occurring agents have shown chemopreventive potential in a variety of bioassay systems and animal models. Flavonoids are constituents of fruits, vegetables, nuts and plant-derived beverages such as tea, wine, and traditional Eastern medicines. Flavonoids are also dietary pharmacological agents that may block neoplastic inception or delay disease progression [4, 5]. Distinct structure-activity relationships (SAR) were also revealed for antioxidant abilities of the flavonoids [6–10]. It is generally accepted that an increase in the number of hydroxyl groups increases the antioxidant activity of flavonoids [11]. In addition, flavonoids are reported to have anti-viral, anti-parasitic, and anti-carcinogenic properties [12]. Reports have indicated that flavonoids inhibit cell growth and induction of apoptosis in many types of cancer cells [13–15]. Moreover, quercetin-mediated apoptosis may result from the disruption of mitochondria and induction of stress signals pathway [16–17].

Recent reports have outlined the mechanisms of proposed anti-obesity [3]. Among them, pre-adipocytes play a key

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Abbreviations: LDH, lactate dehydrogenase; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **ORAC**, oxygen radical absorbance capacity; **PI**, propidium iodide

role in differentiation into mature adipocytes and increase fat mass. Some reports have indicated that flavonoids have been used in cell culture and animal models for treatment of obesity [18 – 21]. Here, we used the murine 3T3-L1 cells line, which is widely used as a cell model. It has been a mainstay for adipose cell biology research over several decades [22 – 24]. Therefore, our study focused on the growth inhibitory effect of flavonoids on 3T3-L1 pre-adipocytes. Moreover, the literature regarding the relationship between the inhibitory effect of 3T3-L1 pre-adipocytes and antioxidant activity is unclear. The aim of this study was to investigate the growth inhibitory effect of flavonoids on 3T3-L1 pre-adipocytes in relation to their antioxidant activity.

2 Materials and methods

2.1 Materials

Flavonoids (naringenin, rutin, hesperidin, resveratrol, naringin, and quercetin), MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], propidium iodide (PI), ribonuclease (RNase), and anti-rabbit or anti-mouse secondary horseradish peroxidase antibodies were purchased from the Sigma Chemical (St. Louis, MO). DMSO was purchased from Merck (Darmstadt, Germany). DMEM, calf serum, and antibiotic mixture (penicillin-streptomycin) were purchased from the Gibco BRL (Grand Island, NY). Anti-capsase-3, anti-PARP, and anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-Bcl-2, anti-Bax, and anti-Bak antibodies were obtained from PharMingen (San Diego, CA). Molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France). PVDF membrane for Western blotting was obtained from Millipore (Bedford, MA). All other chemicals were reagent grade.

2.2 Cell culture

Mouse embryo 3T3-L1 cells (BCRC 60159) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). The culture medium included DMEM, 10% calf serum, 1.5 g/L sodium bicarbonate, and 100 U/mL penicillin-streptomycin. The cell culture condition was 37°C in humidified 5% CO_2 incubator.

2.3 MTT assay

The MTT assay was performed according to the method of Mosmann [25]. The 3T3-L1 pre-adipocytes were plated into 96-well microtiter plates at a density of 1×10^4 cells/well. After 24 h, culture medium was replaced by 200 μL serial dilutions (0 to 1000 μM) of the flavonoids and the cells were incubated for 24, 48, and 72 h. The final concentration of solvent was less than 0.1% in cell culture medium. Culture solutions were removed and replaced by 90 μL culture medium. Ten microliters of sterile filtered MTT solution (5 mg/mL) in PBS (PBS, 0.85% NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4 and 1.76 mM KH_2PO_4 were dissolved in distilled water, pH 7.4) was added to each well, reaching a final concentration of 0.5 mg MTT/mL. Unreacted dye was removed after 5 h. The insoluble formazan crystals were dissolved in 200 μL/well DMSO and measured spectrophotometrically in a FLUOstar galaxy spectrophotometer (BMG Labtechnologies, Germany) at 570 nm. Inhibition (%) was expressed as the percentage of cell growth compared to control non-treated cells (at which growth is considered 0%) and it was calculated by 100-[$A_{570\ nm}$ (flavonoids)/$A_{570\,nm}$ (control) × 100].

2.4 Oxygen radical absorbance capacity (ORAC) assay

The automated ORAC assay was carried out on a Fluostar Galaxy plate reader (BMG LabTechnologies) with a fluorescent filter (excitation wavelength 540 nm; emission wavelength 565 nm). The procedure was based on a previous report by Cao *et al*. [26] with a slight modification. Briefly, in the final assay mixture, β-PE (16.7 nM) was used as a target of free radical (or oxidant) attack with AAPH (40 mM) as a peroxyl radical generator. Trolox (1 μM) was used as a standard and prepared fresh daily. The analyzer was programmed to record the fluorescence of β-PE every 5 min after AAPH was added. All fluorescence measurements were expressed relative to the initial reading. Final results were calculated using the differences of area under the β-PE decay curves between the blank and a sample and expressed as micromoles of Trolox equivalents per micromoles of sample.

2.5 Cell apoptosis analysis by PI staining

Cell apoptosis was determined by the PI staining method as previously described [27]. The 3T3-L1 pre-adipocytes were stimulated with various concentrations (0, 50, 100 and 250 μM) of quercetin for 24, 48, and 72 h. Briefly, cells were harvested by trypsin-EDTA (TE) solution (0.05% trypsin and 0.02% EDTA in PBS), washed with PBS twice and fixed in 80% ethanol at 4°C for 30 min, followed by an incubation with 100 μg/mL of RNase for 30 min at 37°C. The cells were then stained with 40 mg/mL of PI for 15 min at room temperature and subjected to flow cytometric analysis of DNA content using a FACScan flow cytometric (Becton Dickinson Immunocytometry Systems USA, San Jose, CA). Approximately 1×10^4 counts were made for

each sample. The percentage of distribution of cell apoptosis was calculated by CELL Quest software.

2.6 Lactate dehydrogenase leakage assay

Lactate dehydrogenase (LDH) leakage activity assay was performed using a commercial kit (Sigma Chemical). Cells were incubated with various concentrations (0, 50, 100 and 250 μM) of quercetin for 24 to 72 h and then analyzed for LDH leakage into the culture medium. The total LDH activity was determined after cells were thoroughly disrupted by sonication. The percentage of LDH leakage was then calculated to determine membrane integrity. The LDH leakage was expressed as a percentage of total activity [(activity in the medium)/(activity in the medium + activity of the cells)] × 100.

2.7 Mitochondria membrane potential (ΔΨm) assay

Mitochondria membrane potential (ΔΨm) was determined using the MitoPT™ 100 Test Kit (Immunochemistry Technologies, Bloomington, MN). Cells were seeded in 12-well plates. After 24 h, the cells were treated with various concentrations of quercetin (0, 50, 100 and 250 μM) for 6, 12, and 24 h, respectively. The cells were washed with PBS in 12-well plates, followed by harvesting with 0.1 mL of trypsin-EDTA solution, addition of 1 mL of culture medium, and thorough dispersion. Aliquots of the resultant cell suspensions were placed in Eppendorf tubes at 1×10^6 cells per tube containing 1 mL of culture medium. After centrifugation, cells were incubated with 10 μg/mL JC-1 at 37°C for 15 min in humidified 5% CO_2 incubator. Cells were collected and were washed with 16 assay buffer (MitoPT™ 100 Test kit). The cells were resuspended in an adequate volume of the same solution and analyzed by a FLUOstar galaxy fluorescence plate reader with an excitation wavelength of 485 nm and emission wavelengths at 590 nm for red fluorescence. Apoptotic cells generate a lower reading of red fluorescence, and the changes in the mitochondria membrane potential (ΔΨm) can be most accurately assessed by comparing the red fluorescence of untreated cells and cells treated with quercetin.

2.8 Annexin V-FITC/PI double staining analysis by flow cytometry

Annexin V-FITC/PI double staining of the cells was determined using an Annexin V-FITC kit (ANNEX100F, SEROTEC, UK). To detect early apoptosis, late apoptosis, and necrosis induced by quercetin, 3T3-L1 pre-adipocytes (1×10^6 cells/dish) were added to each well of a 6-cm dish and treated for 72 h at 37°C in 1 mL culture medium containing testing agents at suitable concentrations to give a final concentration of 0, 50, 100 and 250 μM. Approximately 1×10^5 cells were then stained for 10 min at room temperature with Annexin V-FITC and PI in a Ca^{2+}-enriched binding buffer (Annexin V-FITC kit) and analyzed by FACScan flow cytometric. Annexin V-FITC and PI emissions were detected in the FL 1 and FL 2 channels of a FACScan flow cytometric, using emission filters of 525 and 575 nm, respectively. Approximately 1×10^4 counts were made for each sample. The percentages of distribution of normal (Annexin V-FITC−/PI−), early apoptotic (Annexin V-FITC+/PI−), late apoptotic (Annexin V-FITC+/PI+), and necrotic cells (Annexin V-FITC−/PI+) were calculated by CELL Quest software.

2.9 Measurement of caspase-3 activity

Caspase-3 activity assay was performed using a commercial kit (Upstate Biotechnology, Lake Placid, NY). After treatment with quercetin, cells were collected and washed with PBS and lysed in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris HCl, pH 8, 2 mM dithiothione, 10 μg/mL pepstatin A, 2 mM PMSF, and 10 μg/mL leupeptin) for 20 min at 4°C followed by centrifugation ($10\,000 \times g$) for 306 min. The fluorometric substrate (50 μM) and caspase-3 (positive control) or cell lysate sample in 200 μL modified RIPA buffer was added into a 96-well microtiter plate. Fluorescence was measured after 5 min (excitation wavelength, 380 nm; emission wavelength, 460 nm) with a FLUOstar galaxy fluorescence plate reader (BMG LabTechnologies).

2.10 Western blot analysis

The 3T3-L1 pre-adipocytes (1×10^7 cells/10-cm dish) were incubated with quercetin for 24 and 48 h at 37°C in humidified 5% CO_2 incubator. Cells were collected and lysed in ice-cold lysis buffer (20 mM Tris-HCl pH 7.4, 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/mL leupeptin, and 1 mM PMSF). The protein concentration of extracts was estimated with a Bio-Rad DC protein assay (Bio-Rad Laboratories, USA) using BSA as the standard. Total proteins (50–60 μg) were separated by SDS-PAGE using a 12% polyacrylamide gel. The proteins in the gel were transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in PBST (0.05% v/v Tween-20 in PBS, pH 7.2) for 1 h. Membranes were incubated with primary antibody (1 : 5000) at 4°C overnight and then with secondary antibody (1 : 5000) for 1 h. Membranes were washed in PBST for 10 min three times between each step. The signal was detected using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL). The relative

expression of proteins was quantified densitometrically using the software LabWorks 4.5 and was calculated according to the reference bands of β-actin.

2.11 Statistical analysis

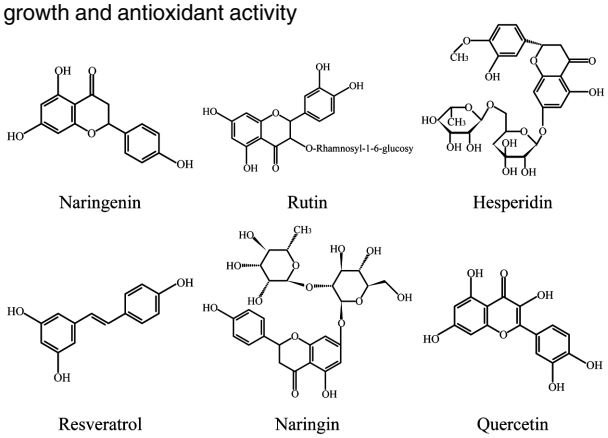
Statistical analysis was performed using SAS software. Analyses of variance were performed using ANOVA procedures. Correlation and regression analyses and principal component analysis were performed using the SigmaPlot scientific graph system. Significant differences ($p < 0.05$) between the means were determined by Duncan's multiple range tests. Each treatment was carried out in triplicate, and the experiment was repeated three times.

3 Results

3.1 Effects of quercetin and other flavonoids on cell population growth and antioxidant activity

Table 1 shows the effect of flavonoids on inhibition of cell population growth and antioxidant activity. Flavonoids including naringenin, rutin, hesperidin, resveratrol and naringin showed lower inhibition (5.6–33.2%) of cell population growth with 50% growth inhibitory concentrations (IC_{50}) values over 500 μM. However, quercetin showed the highest inhibition (71.5%) of cell population growth and with IC_{50} value of 40.4 μM. The ORAC assay measured the capacity of various flavonoids to scavenge peroxyl radicals. Quercetin had the highest $ORAC_{ROO}$ value (Trolox equivalent, 19.9 μM) among the six flavonoids tested. Other flavonoids (naringenin, rutin, hesperidin, resveratrol and naringin) had $ORAC_{ROO}$ values within the range of 12.4–17.0 μM Trolox equivalent. Moreover, flavonoids that inhibited cell population growth were found to have higher antioxidant capacities. As shown in Fig. 1, the effect of quercetin on inhibition of cell population growth in 3T3-L1 pre-adipocytes is evident. The results showed that quercetin caused a significant decrease ($p < 0.05$) in cell population growth of 3T3-L1 pre-adipocytes at 48 and 72 h. However, pre-adipocytes play a key role in differentiation into mature adipocytes and increase fat mass. Our results showed that quercetin played a role in decreased pre-adipocytes population growth. The effect of flavonoids on cell population growth in relation to their $ORAC_{ROO}$ values was evaluated. It was found that there was a significant linear correlation between the influence of flavonoids on cell population growth and their antioxidant activity. A correlation coefficient ($r = 0.80$, $p < 0.01$) was observed between the influences of flavonoids on cell population growth and their $ORAC_{ROO}$ values. These results suggest that the effect of flavonoids tested on cell population growth was well correlated with their antioxidant activity.

Table 1. Effect of flavonoids on inhibition of cell population growth and antioxidant activity



Flavonoids	Inhibition (%)[a)]	ORAC[b)]
Naringenin	28.3 ± 1.2[c]	12.4 ± 1.2[d]
Rutin	8.1 ± 0.5[d]	13.8 ± 1.2[cd]
Hesperidin	11.1 ± 0.9[d]	14.7 ± 1.5[c]
Resveratrol	33.2 ± 5.4[b]	17.0 ± 1.2[b]
Naringin	5.6 ± 0.6[d]	12.4 ± 0.4[d]
Quercetin	71.5 ± 5.2[a]	19.9 ± 1.5[a]

a) Cells were incubated with 100 μM of flavonoids for 72 h.
b) ORAC value was expressed as μM of Trolox equivalents per μM of sample. Reported values are the means ± SD ($n = 3$). Values bearing different superscript letters are significantly different at $p < 0.5$.

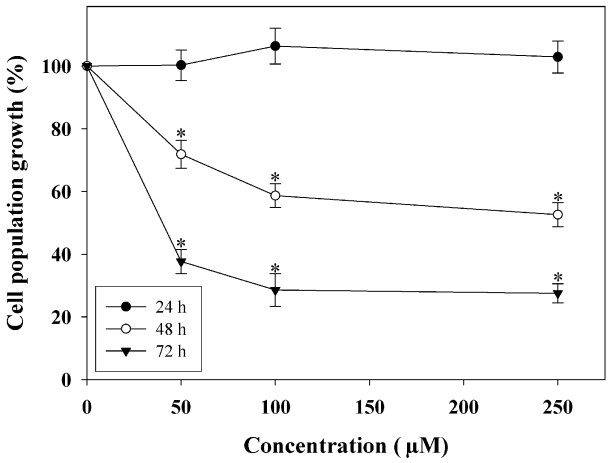


Figure 1. Effect of quercetin on cell population growth in 3T3-L1 pre-adipocytes. Cells were incubated with 0–250 μM of quercetin for 24–72 h at 37°C in humidified 5% CO_2 incubator.

3.2 Effect of quercetin on induction of cell apoptosis

To quantify the degree of apoptosis, the amount of sub-G_1 DNA was analyzed by flow cytometry. Figure 2 shows the effect of quercetin on induction of apoptosis in 3T3-L1 pre-

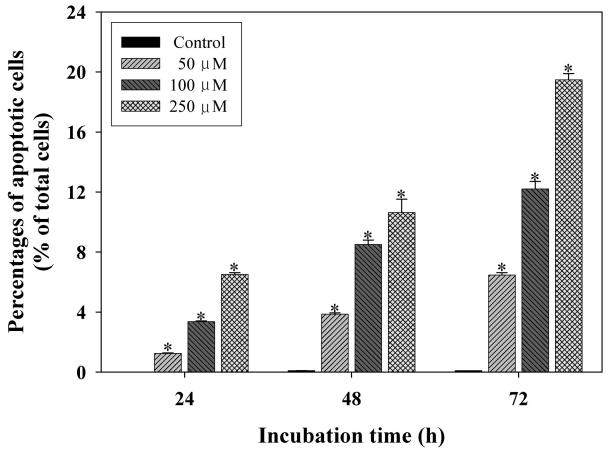


Figure 2. Flow cytometric analysis of quercetin-mediated cell apoptosis in 3T3-L1 pre-adipocytes. The ratio of apoptotic cells was calculated by CELL Quest software (means ± SD, $n = 3$). *$p < 0.05$ significantly different to control.

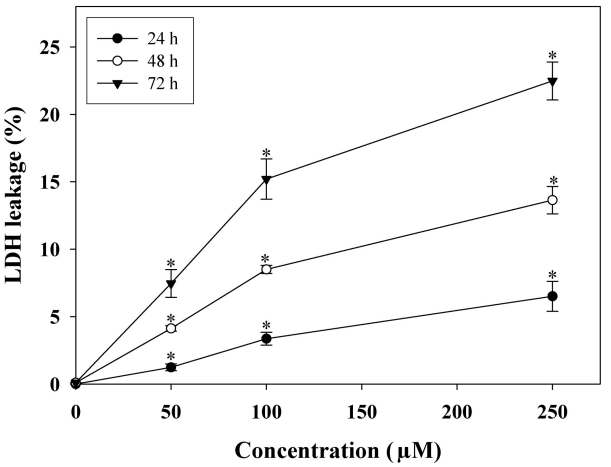


Figure 3. Effect of quercetin on LDH leakage in 3T3-L1 pre-adipocytes. The LDH leakage was examined by detecting the LDH activity in the culture medium. Reported values are the means ± SD ($n = 3$). *$p < 0.05$ significantly different to control.

adipocytes. The 3T3-L1 pre-adipocytes were treated with various concentrations of quercetin for 24, 48, and 72 h. Flow cytometric analyses of 3T3-L1 pre-adipocytes exposed to quercetin showed that the increase of apoptotic cells was time and dose dependent. Thus, treatment of 3T3-L1 pre-adipocytes with quercetin caused cell apoptosis.

3.3 LDH leakage of cells by quercetin

The data indicated that quercetin treatment of 3T3-L1 pre-adipocytes caused inhibition of population growth and cell apoptosis. To double check our data, a quantitative analysis of LDH activity was used to determine the percentage of dead cells. As shown in Fig. 3, treatment with quercetin caused a significant increase ($p < 0.05$) in LDH leakage as compared to the control.

3.4 Collapse of mitochondria membrane potential

Figure 4 shows the effect of quercetin on mitochondria membrane potential in 3T3-L1 pre-adipocytes. Cells were treated with various concentrations of quercetin for 6–24 h. The results showed a reduction of fluorescence intensity with the increase of treatment time and concentrations, reflecting the collapse of mitochondria membrane potential.

3.5 Apoptosis/necrosis induced by quercetin

Analysis of the cell populations showed different sets of populations, wherein Annexin V-FITC–/PI+ was designated as control, Annexin V-FITC+/PI– were designated as early apoptotic, Annexin V-FITC+/PI+ were designated as late apoptotic, and Annexin V-FITC–/PI+ were designated as necrotic. As shown in Fig. 5, cells were treated with various concentrations of quercetin for 72 h. The results indicate that treatment with quercetin decreased the number of normal cells in a dose-dependent manner. The number of apoptotic cells, including early apoptotic (Annexin V-FITC+/PI–) and late apoptotic cells (Annexin V-FITC+/PI+), increased in a dose-dependent manner. When the treatment concentrations increased, the percentages of normal cells decreased from 100% (control) to 68.2% (250 μM). The percentages of apoptotic cells (including

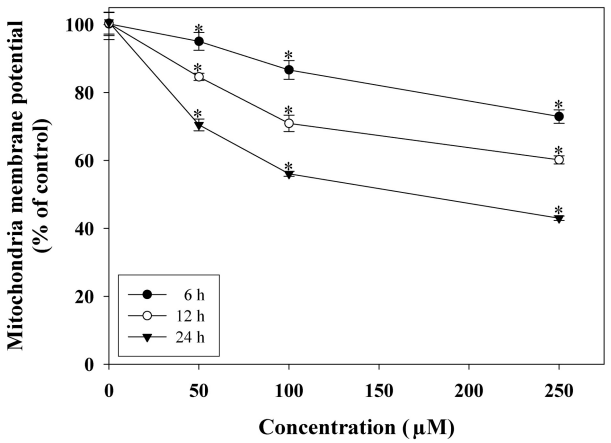


Figure 4. Effect of quercetin on mitochondria membrane potential (ΔΨm) in 3T3-L1 pre-adipocytes. Results are expressed as percentages of mitochondria membrane potential as compared with untreated control (means ± SD, $n = 3$). *$p < 0.05$ significantly different to control.

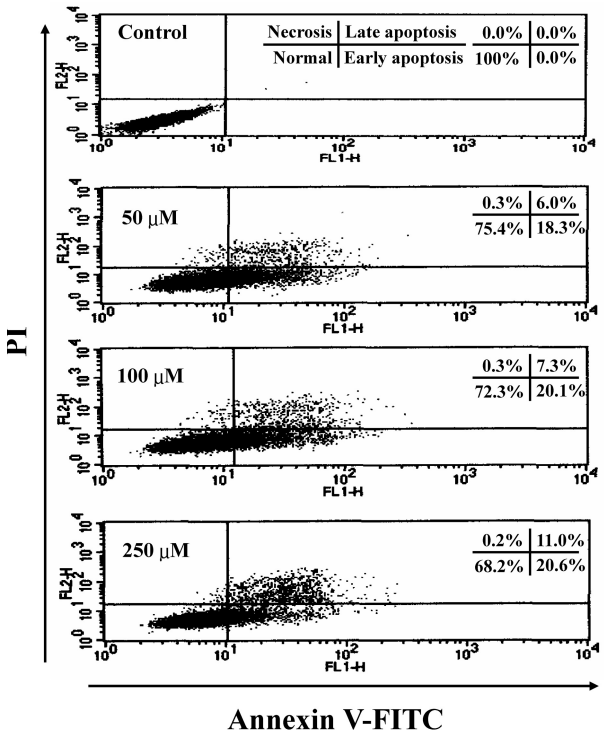


Figure 5. Apoptosis and necrosis induced by quercetin in 3T3-L1 pre-adipocytes. Flow cytometric analysis of annexin V-FITC/PI double stained cells. Cells were untreated or treated by quercetin with 0, 50, 100 and 250 μM for 72 h. The percentages of cells were calculated by CELL Quest software (means, $n = 3$).

early apoptotic and late apoptotic) increased from 0% (control) to 31.6% (250 μM).

3.6 Modulation of apoptosis-related activity and proteins by quercetin

The effect of quercetin on caspase-3 activity in 3T3-L1 pre-adipocytes is shown in Fig. 6. The results indicate that quercetin treatment caused a significant time- and dose-dependent increase in the caspase-3 activity ($p < 0.05$). Additional evidence of caspase-3 activation is the proteolysis of procaspase-3 into small active fragments and cleavage of PARP. To determine the effect of quercetin on apoptotic pathways, transcription factor caspase-3 and PARP protein expression were evaluated by Western blot analysis. Effects of quercetin on caspase-3 and PARP protein levels in 3T3-L1 pre-adipocytes are shown in Fig. 7. Quercetin stimulated caspase-3 expression in a time- and dose-dependent manner with a significant effect ($p < 0.05$) and maximal increase 3.26-fold after treatment with 250 μM of quercetin for 48 h. Treatment with quercetin in cells induced PARP down-regulation in a time- and dose-dependent manner with a significant effect ($p < 0.05$) and maximal degradation 0.5-fold

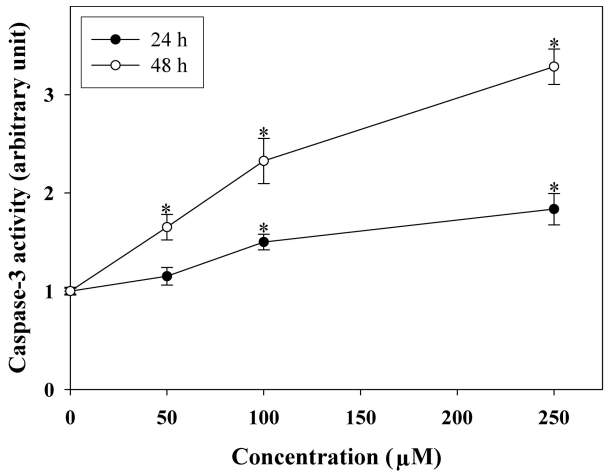


Figure 6. Effect of quercetin on caspase-3 activity in 3T3-L1 pre-adipocytes. Cells were incubated with 0 to 250 μM of quercetin for 0–48 h. Reported values are the means ± SD ($n = 3$). *$p < 0.05$ compared with control.

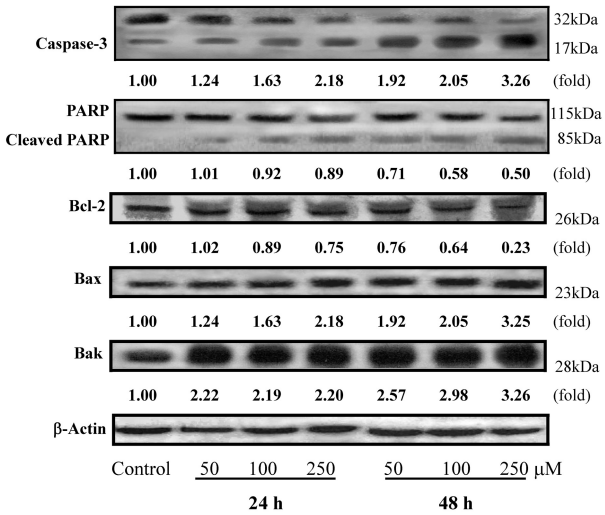


Figure 7. Effects of quercetin on caspase-3, PARP, Bcl-2, Bax, and Bak protein levels in 3T3-L1 pre-adipocytes. 3T3-L1 pre-adipocytes were treated by quercetin with 50, 100, and 250 μM for 24 and 48 h at 37°C in humidified 5% CO_2 incubator. Protein level was analyzed by Western blot analysis. The relative expression of proteins was quantified densitometrically using the software LabWorks 4.5 and calculated according to the reference bands of β-actin.

after treatment with 250 μM of quercetin for 48 h. Effects of quercetin on Bcl-2, Bax, and Bak protein levels in 3T3-L1 pre-adipocytes are also shown in Fig. 7. Cells treated with quercetin induced Bcl-2 down-regulation in a time- and dose-dependent manner with a significant effect ($p < 0.05$) and maximal down-regulation 0.23-fold after treatment with 250 μM of quercetin for 48 h. Quercetin stimulated Bax and Bak expression in a time- and dose-dependent manner with a significant effect ($p < 0.05$) and maxi-

mal increase of 3.25- and 3.26-fold, respectively, after treatment with 250 μM of quercetin for 48 h.

4 Discussion

The results of the MTT assay in the present study clearly indicate that quercetin induced inhibition of cell population growth of 3T3-L1 pre-adipocytes. Previous studies indicated that quercetin induced growth inhibition and cell death in cancer cells [28, 29]. Many studies in different cell lines, animal models, and human epidemiological trials have shown the potential of dietary polyphenols as anti-proliferation agents [30, 31].

Polyphenols affect cell functions such as growth, differentiation and apoptosis [32 – 34]. Many of the biological properties of flavonoids may be related, partially at least, to their antioxidant and free radical scavenging ability [10]. Our data indicate that quercetin had the highest $ORAC_{ROO}$ value (Trolox equivalent, 19.9 μM) among the six flavonoids tested (Table 1). The numbers of hydroxyl groups suggest that additional hydroxyl groups make phenols better antioxidants. It has been reported that quercetin (3,3′,4′,5,7-pentahydroxyflavone) has higher antioxidant activity [35]. Moreover, it was found that there was a significant correlation between the influence of flavonoids on both forms of cell population growth and their $ORAC_{ROO}$ value ($r = 0.80$, $p < 0.01$). These results suggest that flavonoids tested on cell population growth were well correlated with their antioxidant activity. The data indicate that quercetin had the highest inhibition of population growth among the six flavonoids tested.

The treatment of 3T3-L1 pre-adipocytes with quercetin increased the induction of cell apoptosis in a time- and dose-dependent manner (Fig. 2). The inhibition of proliferation and induction of apoptosis are regulated by a network of signaling pathways and transcription factors, which are possible targets for rational tumor therapy [36, 37]. The goal of cell apoptosis is to inhibit pre-adipocytes, because the increase in number and size of adipocytes was differentiated from fibroblastic pre-adipocytes in adipose tissues. Moreover, a quantitative analysis of LDH activity can determine the percentage of dead cells. Our results indicate that quercetin exhibited cytotoxicity against 3T3-L1 pre-adipocytes in a time- and dose-dependent manner. The results of the LDH assay (Fig. 3) were similar to the flow cytometry results (Fig. 2) for cell apoptosis (PI staining method). Detection of the mitochondrial membrane potential event provided an early indication of the initiation of cellular apoptosis. In general, changes in the membrane phosphotidylserine externalization are generally observed at a later stage than the loss of mitochondria membrane potential [38]. In the present study, 3T3-L1 pre-adipocytes treated with quercetin increased the loss of mitochondria membrane potential in a time- and dose-dependent manner (Fig. 4). Annexin V-FITC binds to phosphatidylserine and can be used to detect the early stages of apoptosis [39]. PI could not enter cells with intact membranes and was used to differentiate between the normal (Annexin V-FITC−, PI−), early apoptotic (Annexin V-FITC+, PI−), late apoptotic cells (Annexin V-FITC+, PI+), and necrotic cells (Annexin V-FITC−, PI+). We postulated that Annexin V would discriminate between non-apoptotic and apoptotic cells and could be used to develop a flow cytometric assay for apoptosis. Exposing the 3T3-L1 pre-adipocytes treated with quercetin increased the early apoptotic cell population in a dose-dependent manner (Fig. 5). These results demonstrated that quercetin could induce apoptotic cell death in 3T3-L1 pre-adipocytes.

In an attempt to elucidate the molecular mechanisms underlying quercetin-induced apoptosis of 3T3-L1 pre-adipocytes, we determined the protein levels of various key apoptosis-linked gene products, caspase-3, PARP, Bcl-2, Bax, and Bak (Fig. 7). Mounting evidence suggests that activation of caspases trigger the apoptotic process in various cells [40]. Caspase-3, a member of the caspase family, has been shown to play an essential role in apoptosis induced by a variety of stimuli [41, 42]. Our study indicated that quercetin stimulated caspase-3 expression and then caspase-3 protein may play a key role in quercetin-induced apoptosis in 3T3-L1 pre-adipocytes. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. This cleavage leads to its inactivation, thus preventing the futile DNA repair cycle. Subsequent Western blot analysis disclosed progressive proteolytic cleavage of PARP in 3T3-L1 pre-adipocytes after treatment with quercetin. Some members of Bcl-2 family, such as Bcl-2, function as cell-death suppressors, and other homologues, including Bax and Bak, have powerful death promoting abilities. It was well known that the proteins of the Bcl-2 family play a pivotal role in cells undergoing apoptosis by interfering with the caspases [43, 44]. It was proposed that the ratio between Bcl-2 and Bax is more important in the regulation of apoptosis than the level of each protein separately [45]. The ratio of Bcl-2 to Bax, rather than the levels of the individual proteins, is considered critical in determining the survival/death of cells [46, 47]. Moreover, Bax overexpression has been demonstrated to accelerate apoptotic cell death [48]. Our study indicated that treatment with quercetin markedly decreased Bcl-2 protein and increased Bax and Bak protein expression, suggesting that the decreased level of anti-apoptotic Bcl-2 protein and increased level of pro-apoptotic Bax and Bak proteins may play a key role in quercetin-induced apoptosis in 3T3-L1 pre-adipocytes. The inhibition of quercetin on intracellular triglycerides in 3T3-L1 adipocytes was 44.6% after treatment with 250 μM of quercetin for 72 h (data not show). It has been reported that quercetin

and rutin may promote body mass reduction in obese patients [49]. Our unpublished data indicate that intake of quercetin could be beneficial for the suppression of high fat diet-induced obesity in rats.

In this study, we have clearly shown that quercetin can promote induction of apoptosis in 3T3-L1 pre-adipocytes. The overall effect of flavonoids tested on cell population growth was well correlated with their antioxidant activity of ORAC value. Furthermore, our results indicated that the growth-inhibitory property of quercetin was mainly due to the induction of apoptosis as evidenced by collapse of mitochondria membrane potential, down-regulation of PARP and Bcl-2, and activation of caspase-3, Bax, and Bak. These results demonstrate that quercetin efficiently induces apoptosis in 3T3-L1 pre-adipocytes.

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5 References

[1] Rodriguez, A., Duran, A., Selloum, M., Champy, M. F., *et al*., *Cell. Metab.* 2006, *3,* 211 – 222.

[2] Kopelman, P. G., *Nature* 2000, *404*, 635 – 643.

[3] Wang, Y. W., Jones, P. J., *Int. J. Obes. Relat. Metab. Disord.* 2004, *28*, 941 – 955.

[4] Gao, Z., Huang, K., Yang, X., Xu, H., *Biochim. Biophys. Acta* 1999, *1472*, 643 – 650.

[5] Wong, W. S., McLean, A. E., *Toxicology* 1999, *139*, 243 – 253.

[6] Hu, J. P., Calomme, M., Lasure, A., DeBruyne, T., *et al*., *Biol. Trace Elem. Res*. 1995, *47*, 327 – 331.

[7] Matthiesen, L., Malterud, K. E., Sund, R. B., *Free Radic. Biol. Med.* 1997, *2*, 307 – 311.

[8] Arora, A., Nair, M. G., Strasburg, G. M., *Free Radic. Biol. Med.* 1998, *24*, 1355 – 1363.

[9] Dugas Jr., A. J., Castaneda-Acosta, J., Bonin, G. C., Price, K. L., *et al*., *J. Nat. Products* 2000, *63*, 327 – 331.

[10] Heim, K. E., Tagliaferro, A. R., Bobilya, D. J., *J. Nutr. Biochem.* 2002, *13*, 572 – 584.

[11] Cao, G., Sofic, E., Prior, R. L., *Free Radic. Biol. Med.* 1997, *22*, 749 – 760.

[12] Ko, W. G., Kang, T. H., Lee, S. J., Kim, Y. C., *et al*., *Phytother. Res.* 2002, *16*, 295 – 298.

[13] Lea, M. A., Xiao, Q., Sadhukhan, A. K., Cottle, S., *et al*., *Cancer Lett.* 1993, *68*, 231 – 236.

[14] Plaumann, B., Fritsche, M., Rimpler, H., Brandner, G., *et al*., *Oncogene* 1996, *13*, 1605 – 1614.

[15] Di Carlo, G., Mascolo, N., Izzo, A. A., Capasso, F., *Life Sci.* 1999, *65*, 337 – 353.

[16] Wang, I. K., Lin-Shiau, S. Y., Lin, J. K., *Eur. J. Cancer* 1999, *35*, 1517 – 1525.

[17] Yoshizumi, M., Tsuchiya, K., Kirima, K., Kyaw, M., *et al*., *Mol. Pharmacol.* 2001, *60*, 656 – 665.

[18] Harmon, A. W., Harp, J. B., *Am. J. Physiol. Cell Physiol.* 2001, *280*, C807 – C813.

[19] Hung, P. F., Wu, B. T., Chen, H. C., Chen, Y. H., *et al*., *Am. J. Physiol. Cell Physiol.* 2005, *288*, C1094 – C1108.

[20] Lin, J., Della-Fera, M. A., Baile, C. A., *Obes. Res.* 2005, *13*, 982 – 990.

[21] Wolfram, S., Wang, Y., Thielecke, F., *Mol. Nutr. Food Res*. 2006, *50*, 176 – 187.

[22] Green, H., Kehinde, O., *Cell* 1974, *1*, 113 – 116.

[23] Spiegelman, B. M., Choy, L., Hotamisligil, G. S., Graves, R. A., *et al*., *J. Biol. Chem.* 1993, *268*, 6823 – 6826.

[24] Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., *et al*., *Science* 2000, *289*, 950 – 953.

[25] Mosmann, T., *J. Immunol. Methods* 1983, *65*, 55 – 63.

[26] Cao, G., Sofic, E., Prior, R. L., *Free Radic. Biol. Med.* 1993, *22*, 749 – 760.

[27] Takada, E., Toyota, H., Suzuki, J., Mizuguchi, J., *J. Immunol.* 2001, *166*, 1641 – 1649.

[28] Gupta, K., Panda, D., *Biochemistry* 2002, *41*, 13029 – 13038.

[29] Ong, C. S., Tran, E., Nguyen, T. T., Ong, C. K., *et al*., *Oncol. Rep.* 2004, *11*, 727 – 733.

[30] Watson, W. H., Cai, J., Jones, D. P., *Annu. Rev. Nutr.* 2000, *20*, 485 – 505.

[31] Matito, C., Mastrorakou, F., Centelles, J. J., Torres, J. L., *et al*., *Eur. J. Nutr.* 2003, *42*, 43 – 49.

[32] Formica, J. V., Regelson, W., *Food Chem. Toxicol.* 1995, *33*, 1061 – 1080.

[33] Plaumann, B., Fritsche, M., Rimpler, H., Brandner, G., *et al*., *Oncogene* 1996, *13*, 1605 – 1614.

[34] Caltagirone, S., Rossi, C., Poggi, A., Ranelletti, F. O., *et al*., *Int. J. Cancer* 2000, *87*, 595 – 600.

[35] Lien, E. J., Ren, S., Bui, H. H., Wang, R., *Free Radic. Biol. Med.* 1999, *26*, 285 – 294.

[36] Thompson, C. B., *Science* 1995, *267*, 1456 – 1462.

[37] Kinloch, R. A., Treherne, J. M., Furness, L. M., Hajimohamadreza, I., *Trends Pharmacol. Sci.* 1999, *20*, 35 – 42.

[38] Raghuvar Gopal, D. V., Narkar, A. A., Badrinath, Y., Mishra, K. P., *et al*., *Toxicol. lett.* 2004, *153*, 201 – 212.

[39] Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C., *J. Immunol. Methods* 1995, *184*, 39 – 51.

[40] Alnemri, E. S., *J. Cell Biochem.* 1997, *64*, 33 – 42.

[41] Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., *et al*., *Nature* 1995, *376*, 37 – 43.

[42] Proter, A. G., Janicke, R. U., *Cell Death. Differ.* 1999, *6*, 99 – 104.

[43] Rao, L., White, E., *Curr. Opin. Gen. Dev.* 1997, *7*, 52 – 58.

[44] Antonsson, B., Martinou, J. C., *Exp. Cell Res.* 2000, *256*, 50 – 57.

[45] Gajewski, T. F., Thompson, C. B., *Cell* 1996, *87*, 589 – 592.

[46] Oltvai, Z. N., Milliman, C. L., Korsmeyer, S. J., *Cell* 1993, *74*, 609 – 619.

[47] Fukamachi, Y., Karasaki, Y., Sugiura, T., Itoh, H., *et al*., *Biochem. Biophys. Res. Commun.* 1998, *246*, 364 – 369.

[48] Kobayashi, T., Ruan, S., Clodi, K., Kliche, K. O., *et al*., *Oncogene* 1998, *16*, 1587 – 1591.

[49] Zielimńska-Przyjemska, M., Dobrowolska-Zachwieja, A., *Pol. Arch. Med. Wewn.* 2005, *113*, 231 – 240.

