Therapeutic effect of quercetin and resveratrol in an in vitro model of Graves' ophthalmopathy

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Therapeutic effect of quercetin and resveratrol in an in vitro model of Graves' ophthalmopathy

Directed by Professor Sang Yeul Lee

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Jin Sook Yoon

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ABSTRACT

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As the management of Graves' ophthalmopathy is challenging and often not satisfactory, a new effective and safe treatment on intractable Graves' ophthalmopathy is mandatory. Flavonoids, which are polyphenolic compounds with a wide distribution throughout the plant kingdom, are found to have anti-inflammatory and anti-adipogenic activities. We investigated the therapeutic effect of quercetin and resveratrol in an *in vitro* model of Graves' ophthalmopathy. Primary cultures of orbital fibroblasts were treated with interleukin-1 (IL-1) β with quercetin or resveratrol in nontoxic concentrations. The inhibitory effect of quercetin and resveratrol on the IL-1β induced proinflammatory cytokine expression and the hyaluronan production by IL-1β or tumor necrosis factor (TNF)-α were determined by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) and by hyaluronan ELISA, respectively. To evaluate anti-adipogenic activities, confluent

fibroblasts were subjected to differentiation protocol including peroxisome proliferator activator gamma (PPARγ) agonist for 10 days, and exposed to quercetin and resveratrol during adipocyte differentiation. Differentiated cells were stained with oil red O, and the expression of PPARγ and CCAAT-enhancer-binding proteins (C/EBP) α, β were determined by western blot. Quercetin attenuated IL-1β (10ng/ml) induced intercellular adhesion molecule-1 (ICAM-1), IL-6, IL-8 and cyclo-oxygenase (COX-2) mRNA expression in a dose- and time- dependent manner. Resveratrol also had a significant suppressive effect on IL-1β induced proinflammatory cytokine expressions. Hyaluronan production was only inhibited by quercetin treatment in a dose- and time-dependent manner. Treatment of both quercetin and resveratrol in the process of adipogenic differentiation inhibited lipid accumulation in the cytoplasm of cells stained with oil red O and resulted in decreased expression of adipogenesis-related factors, PPARγ and C/EBPβ in western blot analyses in a dose-dependent manner. The results suggest that quercetin and resveratrol possess significant anti-inflammatory and anti-adipogenic effects *in vitro*. In addition, quercetin significantly inhibited hyaluronan production. Our study results provided a basis for further study of the potential usage of quercetin and resveratrol for the treatment of Graves' ophthalmopathy.

Key words: adipogenesis, Graves' ophthalmopathy, hyaluronan, inflammation, orbital fibroblasts, quercetin, resveratrol

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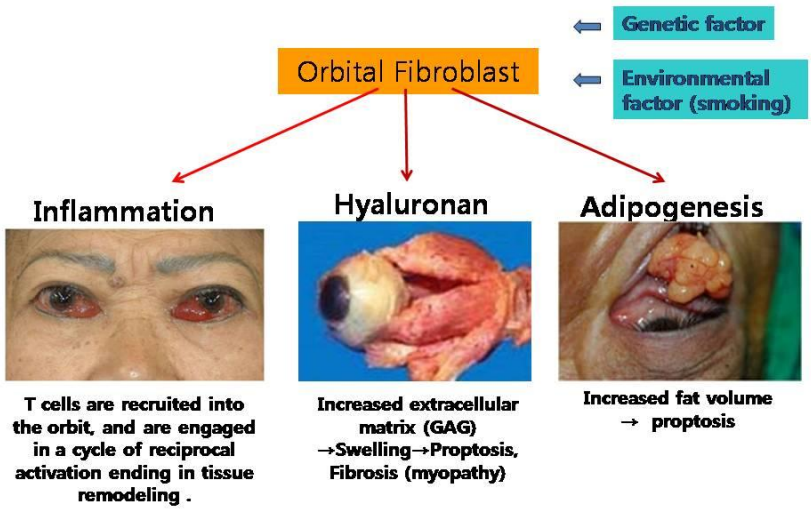
I. INTRODUCTION

Graves' disease is a well-known autoimmune disease of thyroid gland in which autoantibodies bind to thyrotropin receptor on thyroid follicular endothelial cells and thereby activate gland function, leading to excess production of thyroid hormones. Over 25-50% of Graves' disease patients develop manifestation of the eye, known as Graves' ophthalmopathy.[1, 2] Most common features include upper eyelid retraction, edema, and erythema of periorbital tissues, and proptosis. Almost 3-5% of individuals with Graves' ophthalmopathy suffer from intense pain and inflammation, diplopia and sight-threatening compressive optic neuropathy.

Enlargement of extraocular muscle bodies together with an increase of orbital connective / fatty tissues within the bony orbits is responsible for most of the orbital complications in patients with severe active Graves' ophthalmopathy.[3] Tissue

enlargement is affected by marked infiltration of immunocompetent cells, mainly T and B lymphocytes and mast cells, and by abundant collagen and hydrophilic glycosaminoglycans, predominantly hyaluronan. In addition, overabundance of adipose tissue within the orbits is another prominent feature of Graves' ophthalmopathy. It is likely that orbital adipose tissue in Graves' ophthalmopathy is more cellular and comprises a higher proportion of preadipocytes capable of differentiating into adipocytes.[4,5] The process of adipocyte differentiation appears to be phenotypic attribute of orbital fibroblasts, which is not observed in dermal and perimysial fibroblasts (Figure 1). The mechanistic connection of those pathogenic components in Graves' ophthalmopathy is poorly understood. Clinical course of Graves' ophthalmopathy are heterogeneous among patients and often culminates in ocular dysfunction, including restricted eye motility after inflammation subsides.[6]

Figure 1. Main pathologic mechanism of Graves' ophthalmopathy



Glucocorticoids have been used for decades and still are indicated as the first-line treatment due to their anti-inflammatory and immunosuppressive actions, alone or in combination with orbital radiotherapy.[2, 7] However, the management of moderate to severe Graves' ophthalmopathy is challenging, often not satisfactory. They are mostly effective in patients with severe and active eye disease.[7] Soft tissue inflammatory changes, recent onset extraocular muscle involvement, and optic neuropathy are most responsive, while proptosis and longstanding extraocular muscle involvement associated with fibrotic changes are poorly influenced. A major drawback of systemic glucocorticoid therapy is indeed represented by its possible side effects and complications, although transient, cushingoid features, diabetes, depression, reactivation of chronic diseases, infections, hypertension, osteoporosis, increased body weight, peptic ulcer, hirsutism, and cataract, have been reported during

prolonged glucocorticoid therapy for Graves' ophthalmopathy.

Salvi et al. compared rituximab, anti CD 20 antibody with intravenous glucocorticoid therapy in a small, open label nonrandomized pilot study in patients with mild to moderate Graves' ophthalmopathy, but the study was uncontrolled and inadequately powered.[8] Somastatin analogues were initially considered promising treatment for Graves' ophthalmopathy based on nonrandomized open trials.[9] However, this drug was abandoned on the basis of recent randomized prospective trials showing no efficacy.[10, 11] Agents interfering with TNF-α action, which have been used to treat rheumatoid arthritis and inflammatory bowel disease, were found to have efficacy in active Graves' ophthalmopathy in several uncontrolled studies, but there are limitations of high costs and potential side effects.[12, 13] No reliable, specific and safe medical therapeutic agents have yet been developed for Graves' ophthalmopathy. The challenge of developing specific therapies targeting pathways of inflammation, adipose tissue expansion, aberrant accumulation of extracellular matrix macromolecules and fibrosis are mandatory.

Recently, much attention has been focused on natural antioxidants in foods. Flavonoids are a class of natural biological products that are structurally heterogeneous, polyphenolic compounds, present in high concentrations in fruits, vegetables, and other plant-derived foods. It is reported that flavonoids show pharmacologic effects such as antioxidant,[14, 15] antitumor[16] and anti-inflammatory[17] and therapeutic potential for ischemic heart disease and atherosclerosis.[18]

Quercetin (3,3,4,5,7-pentahydroxyl flavonone) is a flavonoid, phytoestrogen, abundantly found in soybeans, vegetables and fruits. Quercetin affects cell cycle kinetics and proliferation and induces apoptosis.[19,20] It has also been found to possess anti-oxidant,[21] anti-inflammatory,[22, 23] and anti-adipogenic effects. [24-26] Resveratrol (3,4',5-trihydroxystilbene) is a naturally occurring phytoalexin found in red wines and grape juice, and has also been reported to have antioxidant,[21] anti-inflammatory,[27] and anticarcinogenic,[28] and anti-adipogenic effects.[24,29]

In this study, we investigated the effect of quercetin and resveratrol on the expression of proinflammatory gene, hyaluronan production induced by interleukin (IL)-1β, and on the adipocyte differentiation in the primary cultures of orbital fibroblasts from patients with Graves' ophthalmopathy.

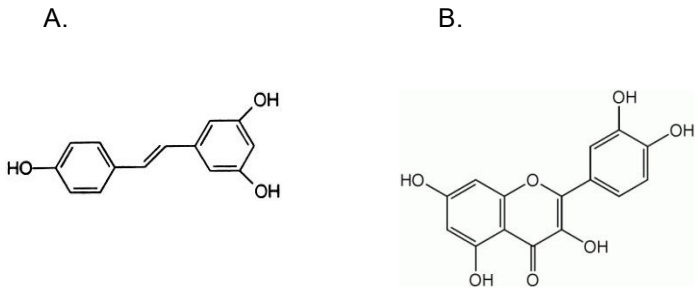
II. MATERIALS AND METHODS

1. Reagents and chemicals

Quercetin (Q 0125), resveratrol (R 5010) were purchased from Sigma-Aldrich, Inc (St. Louis, MO). Structures of the flavonoids used in this study are shown in Figure 2. Dulbecco's modified Eagle's mediam (DMEM), fetal bovine serum (FBS), penicillin, and gentamycin were purchased from Hyclone Laborotaries, Inc (Logan, UT). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, the fluorescent probe propidium iodide (PI) and Oil Red O were purchased from Sigma-Aldrich. Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) cell proliferation assay kit was purchased from Chemicon (Temecular, CA). The annexin V-fluorescence isothiocyanate (FITC) apoptosis kit was purchased from BD Biosciences (Franklin Lakes, NJ). Hyaluronan enzyme-linked immunosorbent assay (ELISA) kit was purchased from Echelon Biosciences (Salt Lake, UT).

Recombinant human (rh) IL-1β and tumor necrosis factor (TNF)-α were purchased from R&D Systems (Minneapolis, MN). Anti-peroxisome proliferator activator gamma (PPARγ) antibody, anti-CCAAT-enhancer-binding proteins (C/EBP) α antibody, anti-C/EBP β antibody and anti-β-actin antibody were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Figure 2. Structure of the quercetin (A) and resveratrol (B).



2. Cell culture and differentiation protocol

Orbital adipose/connective tissue explants were obtained from individuals undergoing surgical decompression for severe Graves' ophthalmopathy (n=5; four women, one man; aged 41-62 years; no smoker). The patients were not on steroid medication for at least three months before surgery, and were euthryoid at the time of culture harvest. Control cells were obtained in the course of orbital surgery for other noninflammatory problems with no history of Graves' ophthalmopathy or Graves' hyperthyroidism (n=5; three women, two men; aged 35-61 years; no smoker). Tissue explants were minced and placed directly in plastic culture dishes, allowing preadipocyte fibroblast to proliferate. Cells were incubated in DMEM containing 20% FBS, penicillin (100 U/mL), and gentamycin (20 μg/mL) in a humidified 5% CO_2 incubator at 37°C, and maintained in two 80-mm flasks with DMEM containing 10% FBS and antibiotics. Monolayers were passaged serially by gently treating with trypsin/ EDTA. The strains were stored in liquid N_2 until needed and used between 3th and 10^{th} passage. Cultured orbital fibroblasts were pretreated with quercetin and resveratrol before incubation with rh IL-1β or TNF-α to study suppressive effect on

inflammation and hyaluronan production.

To initiate differentiation, cells were grown to confluence in six-well plates. Cultures were changed to serum-free DMEM supplemented with 33 μM biotin, 17 μM pantothenic acid, 10 μg/ml transferrin, 0.2 nM T3, 1 μM insulin (Boehringer-Mannheim, Manheim, Germany), 0.2 μM carbaprostaglandin (cPGI2; Calbiochem, La Jolla, CA), and a PPARγ agonist, thiazolidinediones (TZD). Rosiglitazone 10 μM (Cayman) was used in the TZD class of drugs in this study. For the first 4 days only, 10 μM dexamethasone and 0.1 mM isobutylmethylxanthine (IBMX) was included in the media. The differentiation protocol was continued for 10 days, during which the media was replaced every 3-4 days. To evaluate the effect of quercetin and resveratrol on adipocyte differentiation, we exposed cultures to quercetin (10, 50, 100 μM) or resveratrol (10, 30, 50 μM) for the entire 10-day differentiation period.

3. Cell viability and proliferation assay

Cell viability was assessed by using the MTT assay according to the manufacturer's (Sigma-Aldrich) protocol. Briefly, MTT (5 mg/ml) was added to equal 1/10 of the culture volume and incubated for 3 hours. Medium was removed, and the converted dye was solubilized in ice-cold isopropanol. Absorbency of the dye was measured at 560nm with background subtraction at 630 nm on a microplate reader (EL 340 Bio Kinetics Reader; Bio-Tek Instruments, Winooski, VT)

Cell proliferation was determined with a nonisotopic enzyme immunoassay for

BrdU incorporation, following the manufacturer's instruction. Cells were labeled with BrdU for 24 hours. The medium was removed, and cells were fixed with fixing solution at room temperature for 30 minutes. The fixing solution was aspirated and anti-BrdU antibody was added for 1 hour. After washing, 100 µl goat anti-mouse IgG peroxidase conjugate was added for 30 minutes. After repeated washing, 100 µl of substrate was added, and the plate was incubated in the dark for 6 and 24 hours followed by the supply of 100 µl of stop solution. Absorbance was read on a spectrophotometer microplate reader set at dual wavelength of 450/550 nm. The background absorbance of cells receiving no primary antibody was subtracted.

4. Annexin V assay for cytotoxicity

Cells were washed with phosphate buffered saline (PBS) and incubated in serum-free DMEM in the presence of quercetin at 10- 100 µM for 24 hours and resveratrol at 1- 50 µM for 24 hours. An annexin V- FITC kit was used to detect phosphatidylserine externalization, as an index of apoptosis. Cells were washed and incubated for 15 minutes at room temperature in the presence of annexin V labeled with FITC and PI. In total, 10,000 cells were excited at 488 nm, and emission was measured at 530 and 584 nm to assess FITC and PI fluorescence, respectively. Cells were analyzed with a flow cytometer (FACSn; BD Bioscience). Gated cell numbers were plotted on a dot plot with reference to both annexin V and PI staining.

5. Semiquantitative RT-PCR

RNA isolation and semiquantitative reverse transcription polymerase chain reaction (RT-PCR) were performed as described previously. Total RNA was prepared using TriZol (Invitrogen, Carlsbad, CA). cDNA was synthesized from 1 μg of total RNA using 10mM of dNTP mixture (2 μl), recombinant RNasin® ribonuclease inhibitor (0.5 μl), AMV reverse transcriptase (15u), reverse transcription 10X buffer, Oligo$(dT)_{15}$ primer (0.5 μg/μl) (Promega Corporation, Madison, WI).

PCR was performed using 0.25 mM dNTP, 0.25 U Taq polymerase (iNtRON Biotechnology, Inc., Korea), 10 pmole primer pair and 3 μl cDNA with a thermal cycler (PerkinElmer, NY). PCR cycling conditions were as follows: 30 cycles with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), IL-6, IL-8 at 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute, 34 cycles with intercellular adhesion molecule-1 (ICAM-1): 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 1 minute, and 35 cycles with cyclo-oxygenase (COX)-2: 93°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds. Primer sequences specific for amplification genes encoding ICAM-1, IL-6, IL-8, and COX-2 were designed from available human gene sequences (Table 1). The levels of mRNAs, quantified by densitometry scanning of the amplified products electrophoresed on agarose gels, are expressed as the ratio between the density of each gene products and coamplified GAPDH.

Table 1. Primers used for semiquantitative RT-PCR

Target Gene	Primer	Sequence
ICAM-1	forward	5'-GGC CTC AGC ACG TAC CTC TA-3'
	reverse	5'-TGC TCC TTC CTC TTG GCT TA-3'
IL-6	forward	5'-TCA ATG AGG AGA CTT GCC TG-3'
	reverse	5'-GAT GAG TTG TCA TGT CCT GC-3'
IL-8	forward	5'-TTG GCA GCC TTC CTG ATT TC-3'
	reverse	5'-AAC TTC TCC ACA ACC CTC TG-3'
COX-2	forward	5'- GTT CCA CCC GCA GTA CAG-3'
	reverse	5'-GGA GCG GGA AGA ACT TGC-3'
GAPDH	forward	5'-GCC AAG GTC ATC CAT GAC AAC-3'
	reverse	5'-GTC CAC CAC CCT GTT GCT GTA-3'

6. Hyaluronan ELISA

Orbital preadipocyte fibroblasts were grown to confluence in 12-well plates and then incubated for the indicated time periods with variable concentrations of quercetin and resveratrol before stimulation with IL-1β. Supernatants from the cell cultures were collected, and hyaluronan concentrations were determined using a competitive binding hyaluronan ELISA kit (Echelon Biosciences) according to the manufacturer's instructions. The concentration of hyaluronan in the sample was determined using a standard curve generated using known amounts of hyaluronan. Samples were diluted 1:10 before analysis, and the average of triplicate measurements was taken.

7. Oil red O stain

Cells were stained with Oil red O as described by Green and Kehinde.[30] A stock solution of Oil red O (0.5g in 100 ml isopropanol) was prepared and passed through a 0.2 μm filter. To prepare the working solution, 6 ml of the stock solution was mixed with 4 ml of distilled water, left for 1 hour at room temperature, and filtered through a 0.2 μm filter. Cells were washed twice with 1X PBS, fixed with 10% formalin in PBS for 1 hour at 4 °C and stained with 300 μl of the Oil red O working solution for 1 hour at room temperature. The dishes were washed with distilled water before being visualized using Axiovert (Carl Zeiss) light microscope and photographed at X40 and X100 using an Olympus Corp. BX60 light microscope (Olympus, Melville, NY).

8. Western blot assay of PPARγ, C/EBPα, C/EBPβ

Differentiated cells were washed with ice-cold PBS and lysed with cell lysis buffer (20 mM HEPES [pH 7.2], 10% [v/v] glycerol, 10 mM Na3VO4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1% [v/v] Triton X-100; Sigma-Aldrich) on ice for 30 minutes. Lysates were centrifuged for 10 minutes at 12,000 g and cell homogenate fractions stored at -70°C before use.

Protein concentrations in supernatant fractions were determined by the Bradford assay. Equal amounts of protein (50 μg) were boiled in sample buffer and resolved by 10% [w/v] SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF)

membranes (Immobilon; Millipore, Billerica, MA), probed overnight with primary antibodies in TBST, and washed three times with TBST. Anti-PPARγ antibody, anti-C/EBPα antibody, anti-C/EBPβ antibody, and anti-β-actin antibody were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibody and developed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and exposed to X-ray film (Amersham Pharmacia Biotech).

9. Statistical analysis

For the purpose of statistically analyses, each level of mRNAs were quantified by densitometry scanning and expressed as the ratio relative to GAPDH. Each data point represents the mean ± SD of three separate experiments, using cells from three different individuals. Data were analyzed by the Mann-Whitney U test, Student's *t*-test using the SPSS program for Windows, version 12.0.1 (SPSS, Chicago, IL, USA). P-values less than 0.05 were considered to be significant.

III. RESULTS

1. The effect of quercetin and resveratrol on cell viability

The cytotoxicity of quercetin and resveratrol to orbital fibroblasts was detected through the loss of cell viability using MTT assay. More than 95% of cells were viable after challenge with 10-100 μM quercetin for both 6 and 24 h in both normal (Figure 3 A) and Graves' ophthalmopathy (Figure 3B) orbital fibroblasts. Nearly more than 90% of cells were viable after treatment of 10-50 μM resveratrol for both 6 and 24 hours in normal (Figure 4 A) and Graves' ophthalmopathy (Figure 4 B) cells.

Interestingly, treatment of quercetin at low concentrations (10-30 μM) for 24 hours promoted mild proliferation up to 130-140% in both normal and Graves' ophthalmopathy orbital fibroblasts. The cell viability was nearly 100% after treatment of 100 μM quercetin in both normal and Graves' ophthalmopathy cells (Figure 3 A, B), whereas cell viability significantly decreased below 65% after challenge of 100 μM resveratrol for both 6 and 24 hours in both normal and Graves' ophthalmopathy orbital fibroblasts by MTT analysis (Figure 4 A, B).

We could also observe the difference of cell viability according to exposure time of chemicals. Long exposure (24 hours) of compounds at low concentrations induced slightly more proliferation than the short exposure (6 hours) after treatment with both compounds.

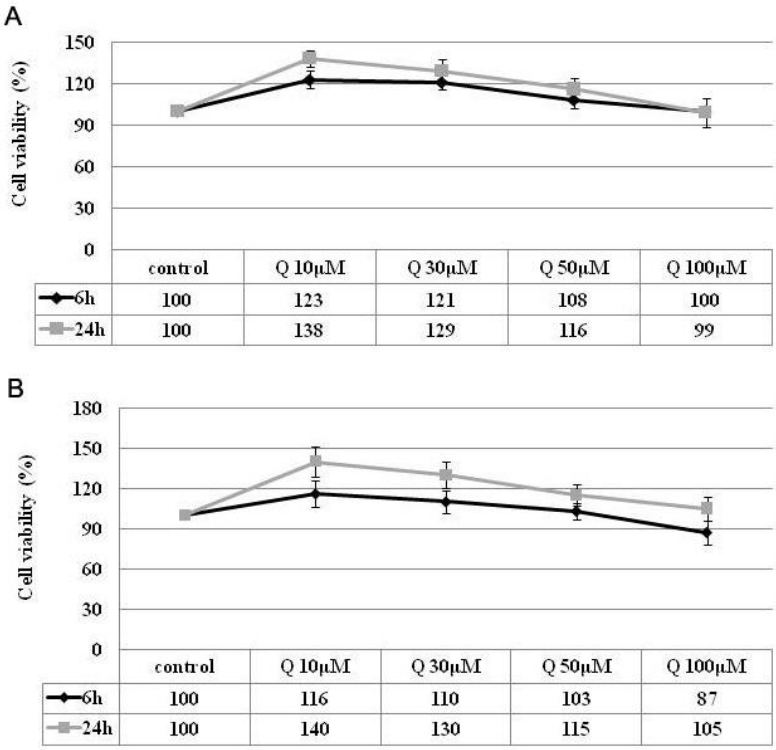


Figure 3. Effect of quercetin on cell viability in normal and Graves' ophthalmopathy orbital fibroblasts. MTT analyses results after the treatment with quercetin in normal (A) and Graves' ophthalmopathy (B) orbital fibroblasts. Data are average of three independent experiments ± SD.

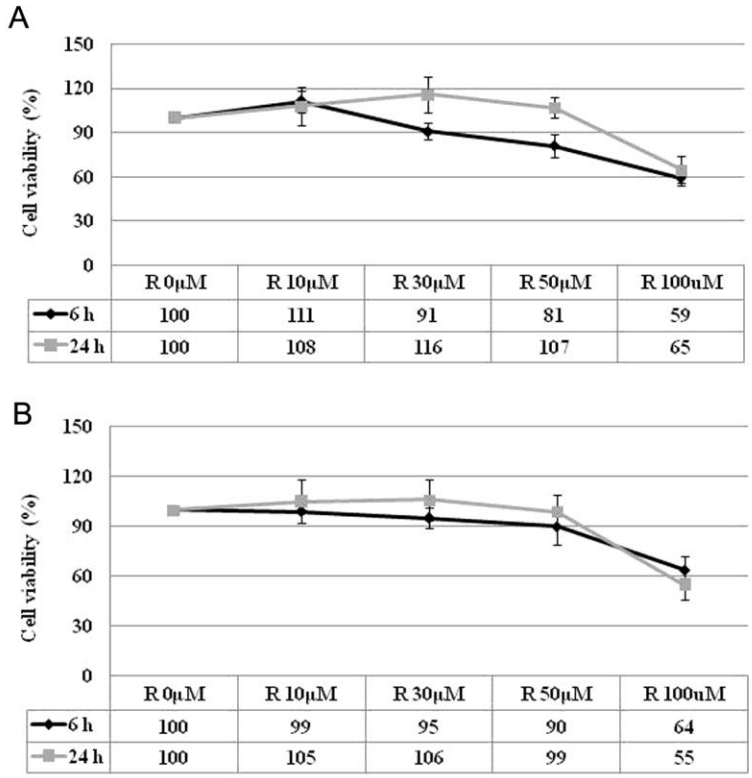
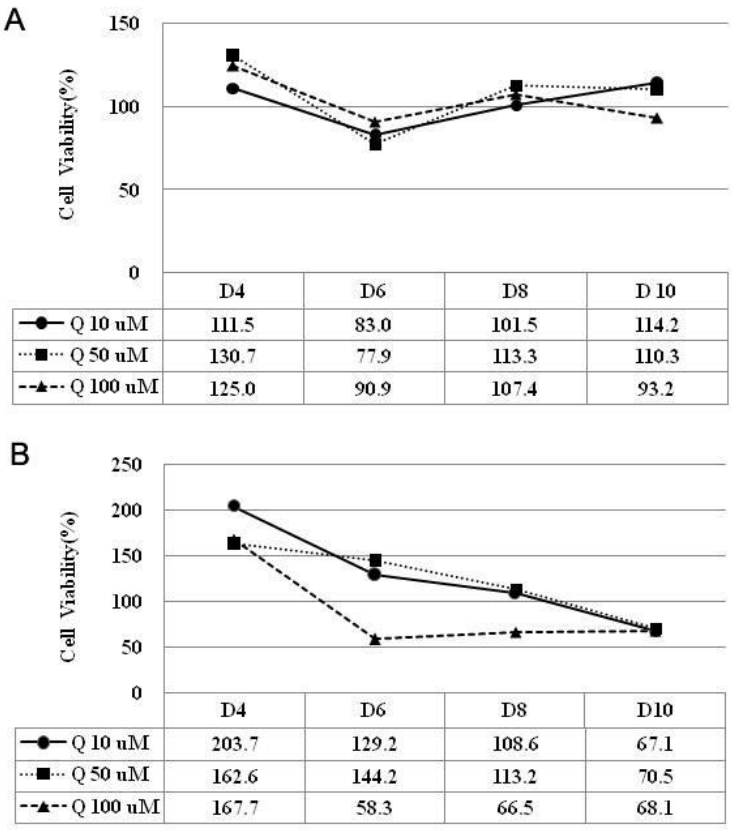


Figure 4. Effect of resveratrol on cell viability in normal and Graves' ophthalmopathy orbital fibroblasts. MTT analyses results after the treatment with resveratrol in normal (A) and Graves' ophthalmopathy (B) orbital fibroblasts. Data are average of three independent experiments ± SD.

Orbital fibroblasts from patient with Graves' ophthalmopathy that were cultured for 10 days in control adipocyte differentiation medium without rosiglitazone and containing rosiglitazone, as described in Materials and Methods. MTT analyses were performed at days 4, 6, 8 and 10 during the process of adipogenic differentiation with continuous challenge of quercetin and resveratrol in orbital fibroblast from Graves' ophthalmopathy patients.

The results of cell viability after challenge with quercetin were significantly different according to the presence of rosiglitazone, an activator of adipogenesis. When rosiglitazone was not added, cell viability was maintained around 100% in all treatment doses (10, 50, 100 μM) throughout 10 days of differentiation (Figure 5A). Slightly decreased viability at day 6 was recovered to nearly 100%. However, when rosiglitazone was contained in the adipogenic medium, treatment of quercetin decreased cell viability continuously (65-70% at day 10) throughout the 10 days of differentiation (Figure 5B).

The MTT results after the treatment of resveratrol was quite different with those treated with quercetin. In the absence of rosiglitazone, the viability seems to recover to 100% in cells exposed to low concentrations (10, 50 μM) of resveratrol at day 10 (Figure 6A). However, cell viability continuously decreased with the challenge of resveratrol during adipogenesis in the presence of rosiglitazone (Figure 6 B). Treatment of resveratrol at high concentration (100 μM) decreased cell viability into 40- 50% at day 10, irrespective of rosiglitazone addition (Figure 6 A, B).

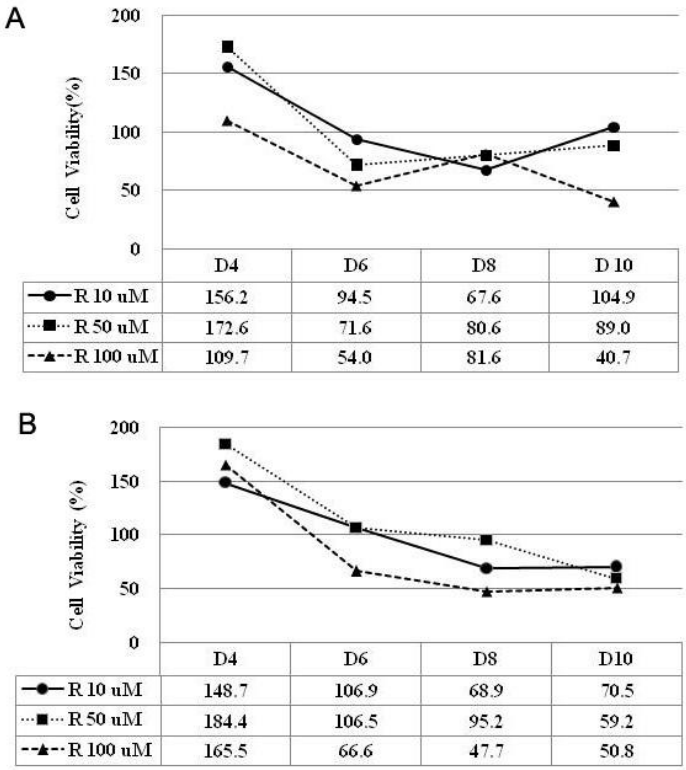


	D4	D6	D8	D 10
Q 10 uM	111.5	83.0	101.5	114.2
Q 50 uM	130.7	77.9	113.3	110.3
Q 100 uM	125.0	90.9	107.4	93.2

	D4	D6	D8	D10
Q 10 uM	203.7	129.2	108.6	67.1
Q 50 uM	162.6	144.2	113.2	70.5
Q 100 uM	167.7	58.3	66.5	68.1

Figure 5. Effect of quercetin on cell viability during adipogenesis.

Orbital fibroblasts from patient with Graves' ophthalmopathy were cultured for 10 days in control adipocyte differentiation medium without rosiglitazone (A) and containing rosiglitazone (B), as described in Materials and Methods. MTT analyses were performed at days 4, 6, 8 and 10 during the process of adipogenic differentiation with continuous challenge of quercetin. Data are average of three independent experiments.



	D4	D6	D8	D 10
R 10 uM	156.2	94.5	67.6	104.9
R 50 uM	172.6	71.6	80.6	89.0
R 100 uM	109.7	54.0	81.6	40.7

	D4	D6	D8	D10
R 10 uM	148.7	106.9	68.9	70.5
R 50 uM	184.4	106.5	95.2	59.2
R 100 uM	165.5	66.6	47.7	50.8

Figure 6. Effect of resveratrol on cell viability during adipogenesis.

Orbital fibroblasts from patient with Graves' ophthalmopathy were cultured for 10 days in control adipocyte differentiation medium without rosiglitazone (A) and containing rosiglitazone (B), as described in Materials and Methods. MTT analyses were performed at days 4, 6, 8 and 10 during the process of adipogenic differentiation with continuous challenge of resveratrol. Data are average of three independent experiments.

As shown in Figure 7, BrdU analyses results were somewhat different from the MTT analyses. Proliferation of normal and Graves' ophthalmopathy orbital fibroblasts was inhibited dose dependently at 6 hours treatment of quercetin, whereas proliferation was promoted at 24 hours treatment of quercetin at low concentration (10-30 μM) up to 180% in normal and 140% in Graves' ophthalmopathy orbital fibroblasts (Figure 7 A, B). In contrast, significant dose dependent-inhibition of proliferation was observed at both 6 and 24 hours of resveratrol treatment in normal orbital fibroblasts (Figure 7 C). In orbital fibroblasts from Graves' ophthalmopathy patients, similar pattern of inhibition of proliferation was observed at 6 hours treatment of resveratrol, and minimally promoted proliferation (up to 118%) was observed at 24 hours treatment of resveratrol at 30 μM concentrations (Figure 7 D). Long exposure (24 hours) of low concentrations (10-30 μM) of quercetin induced mild proliferation of orbital fibroblasts, which results were similar to MTT analyses. Inhibition effects on proliferation of orbital fibroblasts were prominent after the treatment of resveratrol.

To confirm nontoxic concentrations of chemicals in preadipocyte fibroblasts, annexin V-FITC/PI binding assay was performed. The results showed that treatment of quercetin at 10-100 μM and resveratrol at 1-50 μM for 24 hours did not induce significant apoptosis or necrosis in both normal and Graves' ophthalmopathy orbital fibroblasts. Figure 8 shows representative figure from the results in Graves' ophthalmopathy orbital fibroblasts.

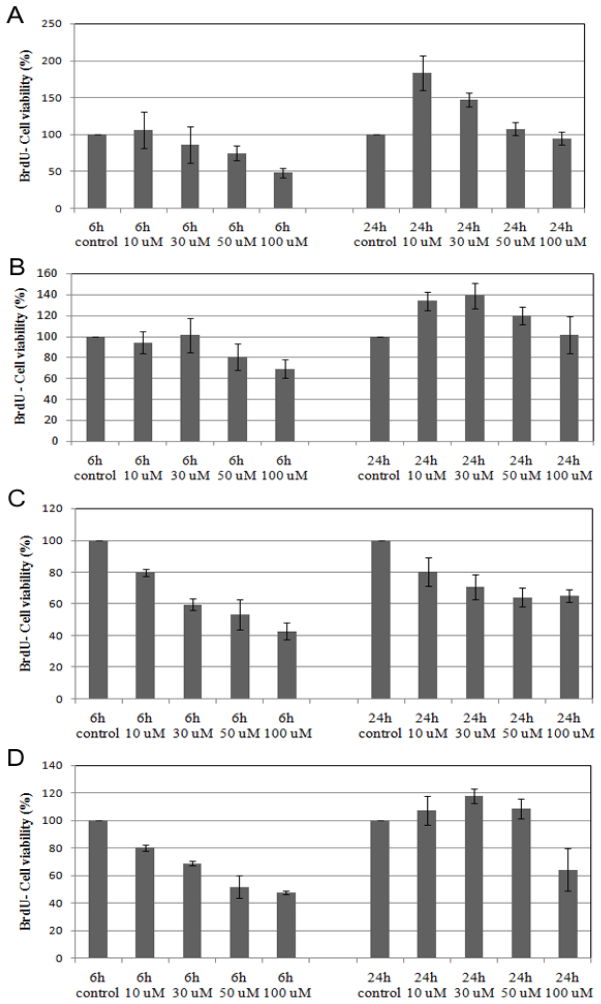


Figure 7. Effects of quercetin and resveratrol on cell proliferation in normal and Graves' ophthalmopathy orbital fibroblasts. BrdU analyses results of orbital fibroblast after treatment with quercetin in normal (A) and Graves' ophthalmopathy (B) orbital fibroblasts, with resveratrol in normal (C) and Graves' ophthalmopathy (D) orbital fibroblasts are shown. BrdU incorporation was expressed as viability (%). Data in the columns represent the mean ± SD of three experiments

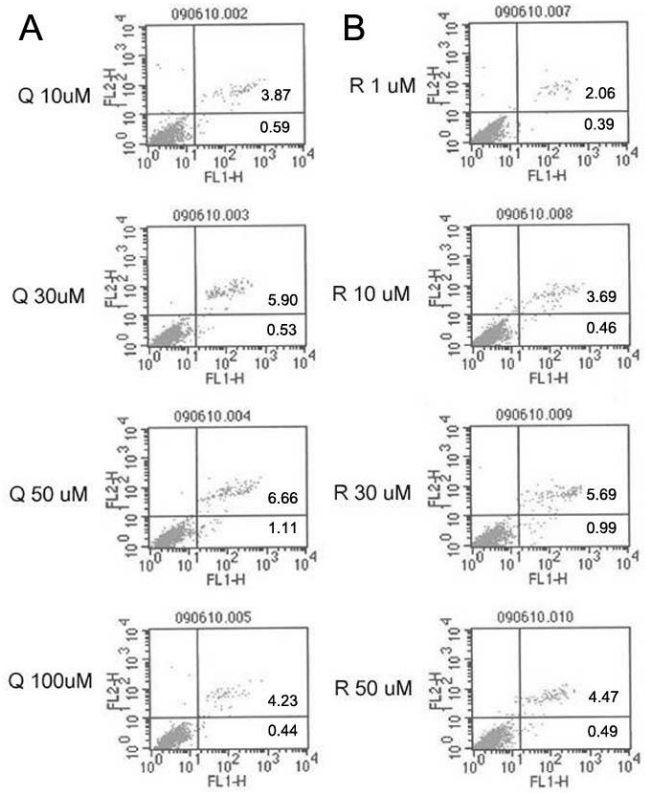


Figure 8. Effect of quercetin and resveratrol on apoptosis in orbital preadipocyte fibroblasts. Graves' ophthalmopathy orbital fibroblasts were incubated with quercetin (Q) at 10-100 μM and resveratrol (R) at 1-50 μM for 24 hours. Next, cells were analyzed using annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining as described in Materials and Methods. LL; viable and undamaged cells (Annexin V^-, PI^-); RL: cells undergoing early apoptosis (Annexin V^+, PI^-); and, RU: necrotic or late apoptotic cells (annexin V^+, PI^+). Each panel shows a typical flow cytometric histogram of 10,000 cells/ sample from a representative experiment.

Therefore, maximal nontoxic concentraion of quercetin and resveratrol was determined as 100 μM and 50 μM, respectively. We determined to use these concentrations as a maximal level to study any suppressive effect of chemicals on the inflammation, hyaluronan production and adipogenesis of orbital fibroblasts from normal and Graves' ophthalmopathy subjects.

2. Determination of IL-1β concentration to study the maximal inhibitory effect of quercetin and resveratrol on the inflammation

As Figure 9 indicates, both pretreatment of quercetin at 100 μM and resveratrol at 50 μM for 6 hours in orbital fibroblasts from Graves' ophthalmopathy induced strong inhibition of ICAM-1 (A), IL-6 (B), IL-8 (C) and COX-2 (D) expression (*P<0.05), when the cells were stimulated with IL-1β at a dose of 10 ng/ml. ICAM-1, IL-6, IL-8, and COX-2 mRNA gene expressions were more significantly induced, and inhibited by quercetin at 100 μM and resveratrol at 50 μM, with the stimulation of 10 ng/ml of IL-1β, in comparison with that of 1 ng/ml of IL-1β. Thus, 10 ng/ml of IL-1β was determined as a stimulation dose for experiments of dose- and time- dependent effect of chemicals.

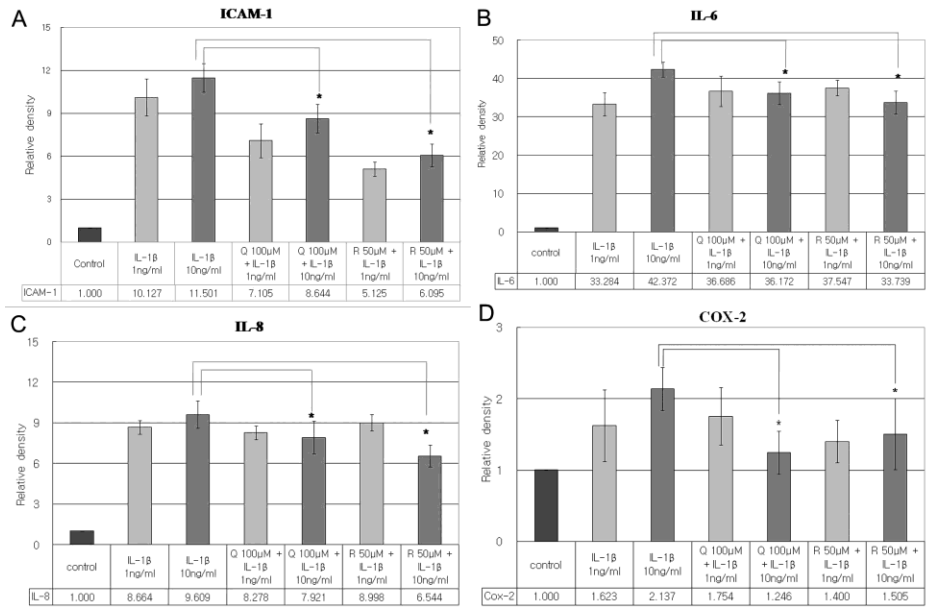


Figure 9. Determination of IL-1β concentration to examine the maximal suppressive effect of quercetin and resveratrol on the expression of proinflammatory cytokines induced by IL-1β in Graves' ophthalmopathy orbital fibroblasts. After pre-treatment of quercetin (100 μM) or resveratrol (50 μM) for 6 hours, cells were treated with IL-1β at 1 ng/ml or 10 ng/ml for 16 hours to detect the effect of chemicals on the expression of ICAM-1, IL-6, IL-8, and COX-2 genes by RT-PCR analyses. Each data were normalized with each GAPDH level, and the relative densities were quantified with a densitometer. Data in the columns represent the mean ± SD of three experiments (in contrast to cells treated with IL-1β only, * $P<0.05$).

3. Inhibition of ICAM-1, IL-6, IL-8 and COX-2 mRNA by quercetin and resveratrol

We investigated the effects of increasing treatment doses and times of quercetin and resveratrol on ICAM-1, IL-6, IL-8 and COX-2 mRNA expression in response to IL-1β challenge of orbital fibroblasts from Graves' ophthalmopathy patients. We chose three concentrations of quercetin (10, 50, and 100 μM) and resveratrol (10, 30, and 50 μM) for 6 hours before treatment with IL-1β (10 ng/ml) for 16 hours to study their dose-dependent effects. Also, to study time-dependent effects, cells were treated with quercetin (100 μM) and resveratrol (50 μM) for 3, 6, 9, and 24 hours before treatment with IL-1β (10 ng/ml) for 16 hour.

As shown in Figure 10 and 11, pretreatment of quercetin showed a significant inhibition of ICAM-1, IL-6, IL-8 and COX-2 mRNA expression induced by IL-1β in a dose- and time- dependent manner. Resveratrol also had a significant suppressive effect on IL-1β induced proinflammatory cytokine expressions in mRNA levels (Figure 12). The inhibitions of IL-8 and COX-2 mRNA expression by resveratrol were dose dependent. Time-dependent manners of inhibition were not observed after the treatment of resveratrol (data not shown).

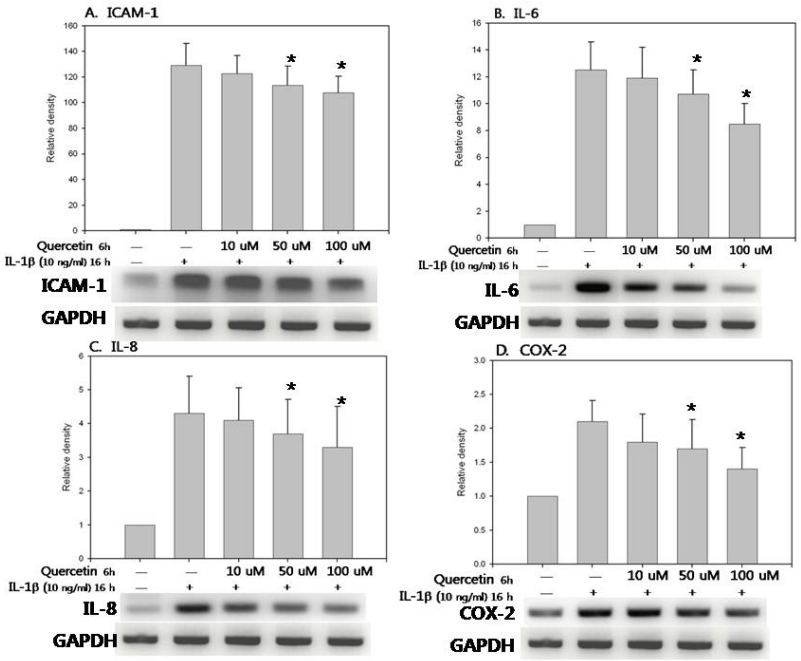


Figure 10. Effects of various concentrations of quercetin on IL-1β induced ICAM-1, IL-6, IL-8 and COX-2 mRNA expression. Orbital fibroblasts from Graves' ophthalmopathy were either not pretreated or pretreated with various concentrations (10, 50, and 100 μM) of quercetin for 6 hours, then washed twice with PBS. Cells were then stimulated with IL-1β (10ng/ml) for 16 hours. Total RNA was isolated and analyzed by RT-PCR as described in Materials and Methods. Each data were normalized with each GAPDH level, and the relative densities were quantified with a densitometer. Results were representative of three separate experiments. Data in the columns represent the mean ± SD of three experiments as to ICAM-1 (A), IL-6 (B), IL-8 (C) and COX-2 (D) (in contrast to cells treated with IL-1β only, * $P<0.05$).

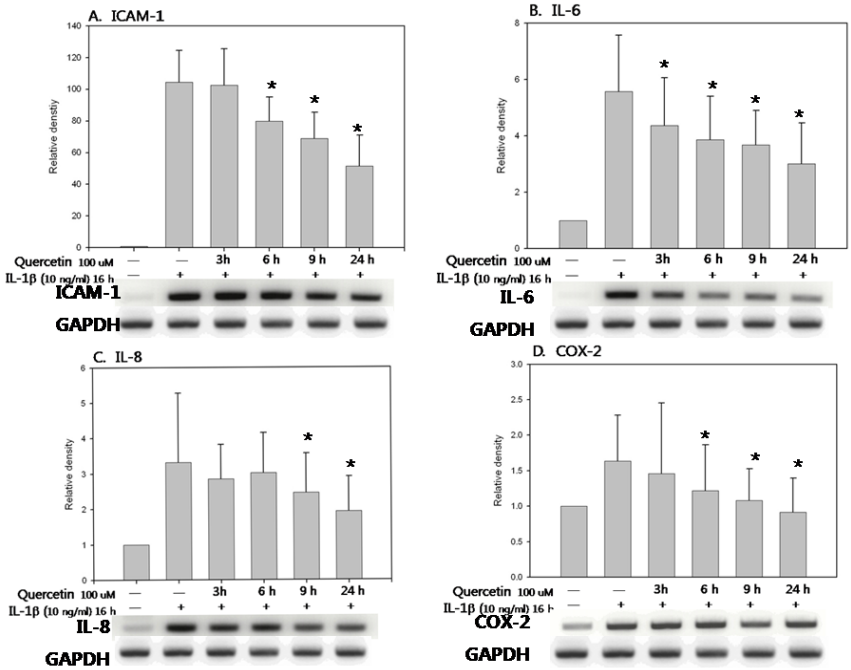


Figure 11. Effects of quercetin for various duration of treatment on IL-1β induced ICAM-1, IL-6, IL-8 and COX-2 mRNA expressions. Orbital fibroblasts from Graves' ophthalmopathy was either not pretreated or pretreated with quercetin (100 μM) for various time periods (3, 6, 9, 24 h), and then washed twice with PBS. Cells were then stimulated with IL-1β (10 ng/ml) for 16 hours. Total RNA was isolated and analyzed by RT-PCR as described in Materials and Methods. Each data were normalized with each GAPDH level, and the relative densities were quantified with a densitometer. Results were representative of three separate experiments. Data in the columns represent the mean ± SD of three experiments as to ICAM-1 (A), IL-6 (B), IL-8 (C) and COX-2 (D) (in contrast to cells treated with IL-1β only, * $P<0.05$).

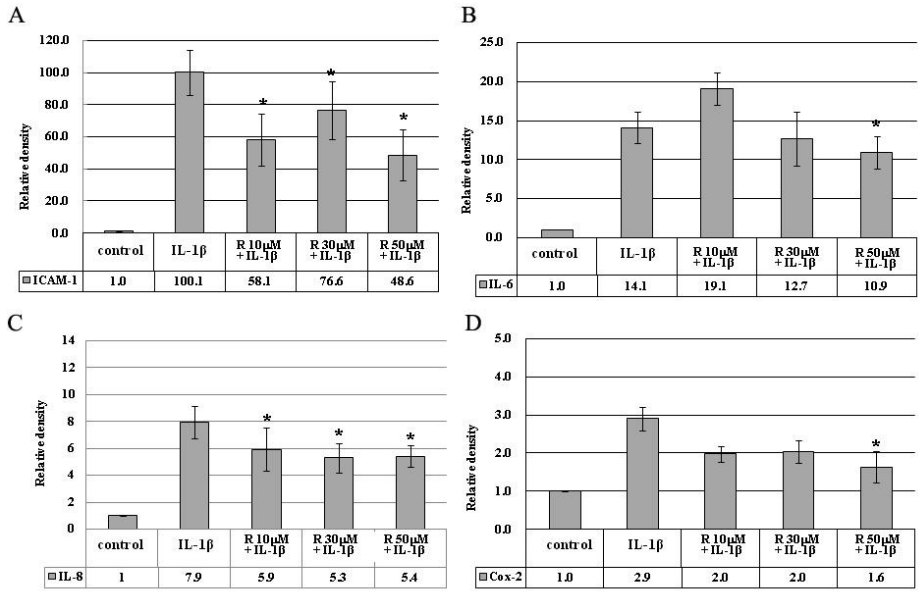


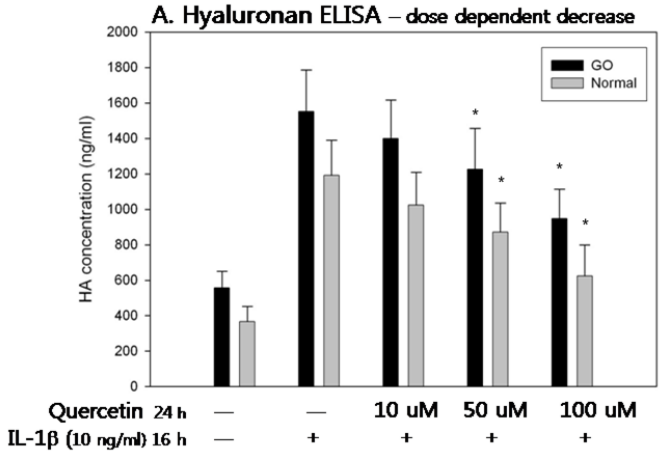
Figure 12. Effects of various concentrations of resveratrol on IL-1β induced ICAM-1, IL-6, IL-8 and COX-2 mRNA expression. Orbital fibroblasts from Graves' ophthalmopathy were either not pretreated or pretreated with various concentrations (10, 30, and 50 μM) of resveratrol for 6 hours, then washed twice with PBS. Cells were then stimulated with IL-1β (10ng/ml) for 16 hours. Total RNA was isolated and analyzed by RT-PCR as described in Materials and Methods. Each data were normalized with each GAPDH level, and the relative densities were quantified with a densitometer. Results were representative of three separate experiments. Data in the columns represent the mean ± SD of three experiments as to ICAM-1 (A), IL-6 (B), IL-8 (C) and COX-2 (D) (in contrast to cells treated with IL-1β only, * $P<0.05$).

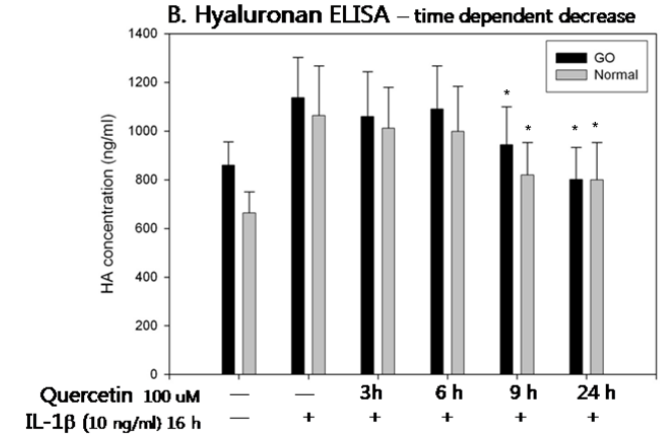
4. Quercetin inhibited hyaluronic acid production in orbital fibroblasts

Orbital fibroblasts produced high level of hyaluronic acid with the stimulation of IL-1β or TNF-α (10 ng/ml, 16 hours) in ELISA analyses. The mean concentration of hyaluronan induced by IL-1β was higher, albeit not significant, in cells from Graves' ophthalmopathy (n=3) than in cells from normal individuals (n=3) (Figures 13 A, B).

Cells were treated with various concentrations (10, 50, 100 μM) of quercetin or resveratrol (10, 30, 50 μM) for 24 hours, before incubation with IL-1β (10ng/ml, 16 hours) for hyaluronan ELISA analyses. Quercetin showed a significant inhibition of hyaluronan production induced by IL-1β in a dose- and time- dependent manner in both normal and Graves' ophthalmopathy orbital fibroblasts (Figures 13 A, B). The inhibition effects of quercetin on the hyaluronan production were similarly observed in a dose-dependent manner in both IL-1β and TNF-α (same condition, 10 ng/ml, 16 hours) stimulated orbital fibroblasts from Graves' ophthalmopathy subjects (Figure 13 C).

However, resveratrol did not show any inhibitory or stimulatory effect on hyaluronan production when stimulated by either IL-1β or TNF-α (data not shown).


A. Hyaluronan ELISA – dose dependent decrease
HA concentration (ng/ml)
GO
Normal
Quercetin 24 h — — 10 uM 50 uM 100 uM
IL-1β (10 ng/ml) 16 h — + + + +


B. Hyaluronan ELISA – time dependent decrease
HA concentration (ng/ml)
GO
Normal
Quercetin 100 uM — — 3h 6 h 9 h 24 h
IL-1β (10 ng/ml) 16 h — + + + + +

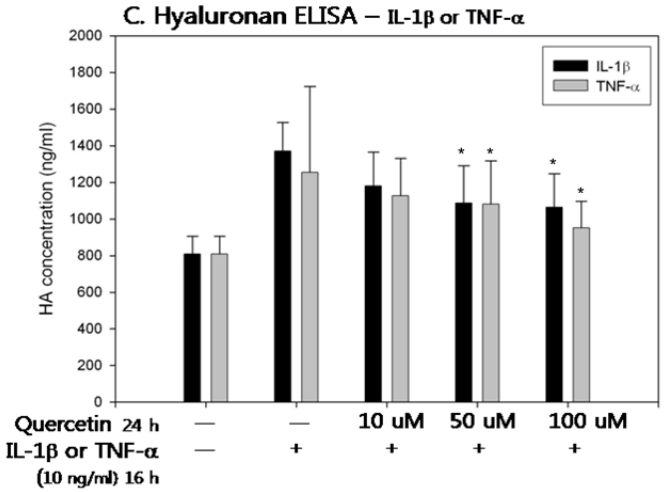

C. Hyaluronan ELISA – IL-1β or TNF-α
HA concentration (ng/ml)
IL-1β
TNF-α
Quercetin 24 h — — 10 uM 50 uM 100 uM
IL-1β or TNF-α — + + + +
(10 ng/ml) 16 h

Figure 13. The effect of quercetin on hyaluronan production induced by IL-1β or TNF-α in orbital fibroblasts from normal and Graves' ophthalmopathy subjects. Hyaluronan levels in supernatants were measured by ELISA. Cells were exposed to quercetin at various concentrations (10, 50, and 100 μM) for 24 hours (A) and to 100 μM of quercetin for various durations (3, 6, 9, 24 hours) (B) before incubation with IL-1β 10 ng/ml for 16 hours. Cells from Graves' ophthalmopathy subjects (n=3) were pretreated with quercetin at various concentrations (10, 50, and 100 μM) for 24 hours and then incubated with IL-1β 10 ng/ml or TNF-α 10ng/ml for 16 hours (C). The average of triplicate measurements was calculated, and the data were expressed as mean ± SD (in contrast to cells not exposed to quercetin, but only to IL-1β or TNF-α, * $P<0.05$).

5. Effect of quercetin and resveratrol on the accumulation of lipid droplets and the expression of transcriptional regulators of adipogenesis

Confluent orbital fibroblasts from Graves' Ophthalmopathy patients were subjected to the differentiated protocol for 10 days. Cells were first examined under light microscopy. Under control adipogenic conditions, preadipocyte fibroblasts lost their stellate fibroblastic appearance and converted to a spherical adipocytic shape, and a fraction of these cells accumulated small lipid droplets (Figure 14 A). Visible from day 3, lipid droplets increased in number and enlarged in size during the 10 days of differentiation. The addition of rosiglitazone (10 μM), a PPARγ agonist significantly increased adipogenesis in light microscopy compared with baseline conditions without rosiglitazone (Figure 14 B). When preadipocyte orbital fibroblasts were cultured under adipogenic condition, and exposed to exogenous IL-1β (10ng/ml) for the first 3 days, accumulation of lipid droplets increased microscopically compared with baseline control conditions (Figure 14 C). Combination of rosiglitazone and IL-1β further stimulated adipogenesis compared with adipogenic condition containing rosiglitazone or IL-1β only (Figure 14 D).

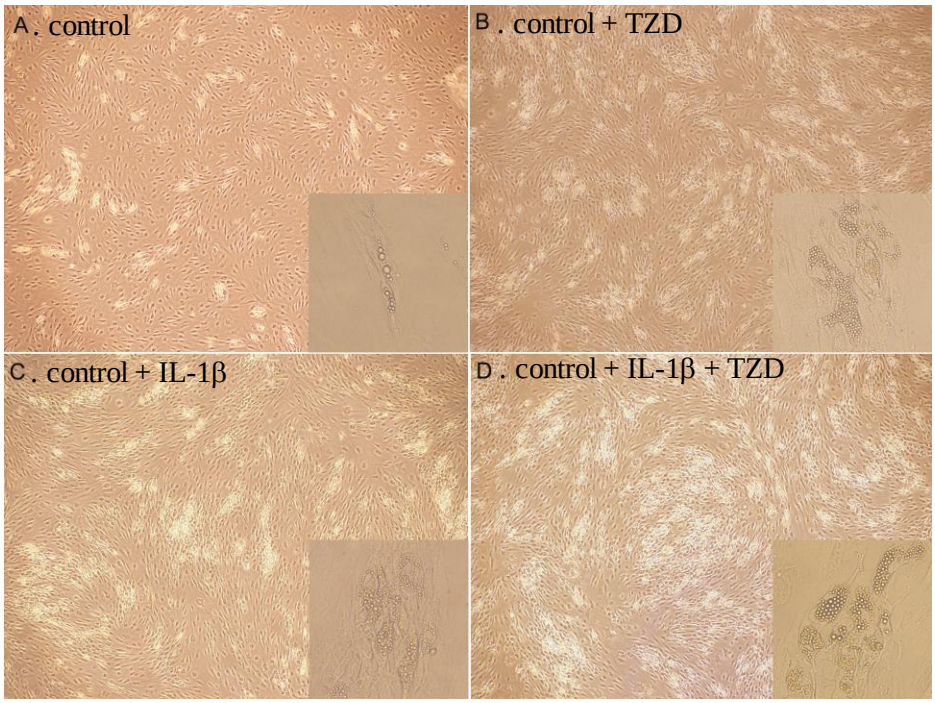


Figure 14. Examination of prestained orbital fibroblasts cultured in adipogenic medium under light microscopy. Orbital fibroblasts from a subject with Graves' ophthalmopathy were cultured in control adipogenic medium (A), adipogenic medium containing rosiglitazone 10 μM for 10 days (B), IL-1β 10ng/ml for first 3 days (C) and both rosiglitazone and IL-1β (D) during differentiation as described in Materials and Method. TZD = Thiazolidinediones (X40, X400: small box).

To examine whether quercetin and resveratrol has any suppressive effects on adipogenesis, those chemicals were added at day 1 in adipogenic medium including rosiglitazone, and continued for the 10 day differentiation period, being replaced whenever media was replaced. Both quercetin and resveratrol decreased number of adipocyte and suppressed accumulation of lipid droplets dose dependently (Figure 15). High power (X400) microscopic examination showed significant reduction of size and number of lipid droplets according to treatment dose of quercetin and resveratrol (Figure 15). Preadipocyte fibroblasts which did not convert into adipocytes were uniform in size and stellate in shape, which maintained viable fibroblastic morphology.

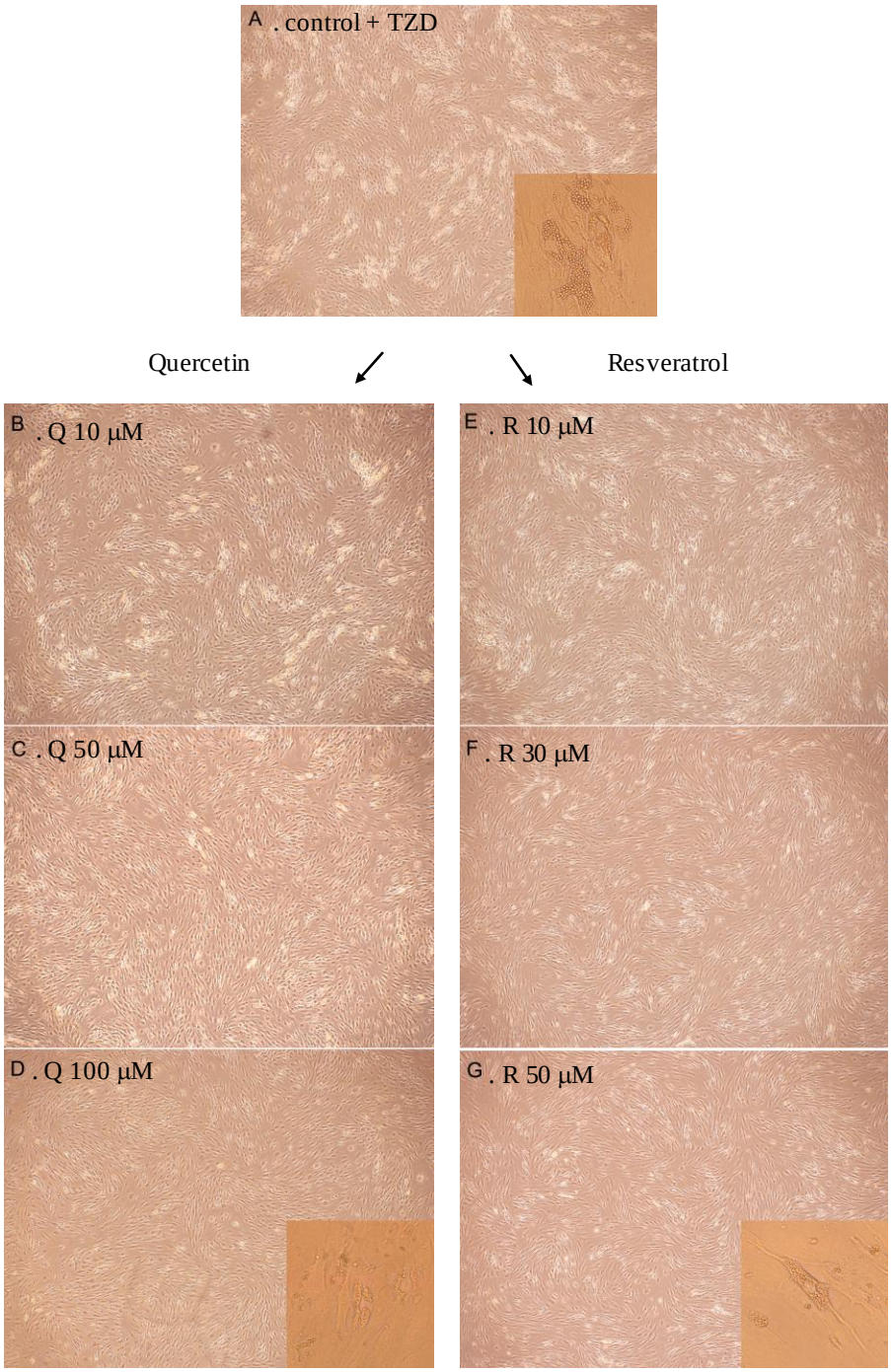

A . control + TZD
Quercetin
Resveratrol
B . Q 10 μM
C . Q 50 μM
D . Q 100 μM
E . R 10 μM
F . R 30 μM
G . R 50 μM

Figure 15. Microscopic examination of prestained cultures grown in adipogenic medium containing either quercetin or resveratrol. Orbital fibroblasts from a subject with Graves' ophthalmopathy were cultured for 10 days in adipogenic medium containing rosiglitazone 10 μM (A). Quercetin (B: 10, C: 50, D: 100 μM) and resveratrol (E: 10, F: 30, G: 50 μM) were exposed to cultures with adipogenic condition in (A) for the entire 10-day differentiation period as described in Materials and Methods. Microscopic examination showed a dose-dependent inhibition of adipogenesis by quercetin and resveratrol. TZD = Thiazolidinediones (X40, X400: small box).

Cells were fixed with 10% formalin for 1 hour and then stained with oil red O to measure lipid accumulation. As shown in Figure 16 and 17, rosiglitazone enhanced, but both quercetin and resveratrol inhibited adipogenesis in a dose dependent manner, as detected by oil red O staining. When the stained cells were examined under microscope with X 400 magnification, significant decrease of cytoplasmic lipid droplets in size and number were observed by treatment of both quercetin and resveratrol, dose dependently.

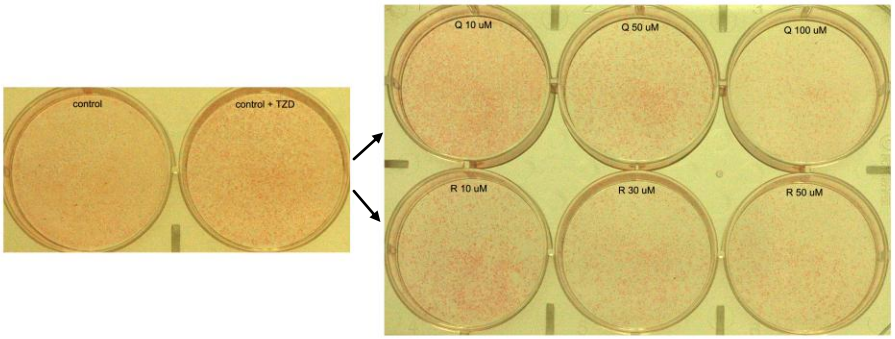


Figure 16. Examination of oil red O stained cultures exposed to quercetin and resveratrol with various concentrations. Orbital fibroblasts from a subject with Graves' ophthalmopathy were cultured for 10 days in control adipogenic medium and containing rosiglitazone 10 μM. Quercetin (10, 50, 100 μM) and resveratrol (10, 30, 50 μM) were exposed to cultures with adipogenic medium containing rosiglitazone for the entire 10-day differentiation. Cells were fixed with 10% formalin for 1 hour and then stained with oil red O to measure lipid accumulation. A dose-dependent inhibition of adipogenesis by quercetin and resveratrol was visible. TZD = Thiazolidinediones (X40, X400: small box).

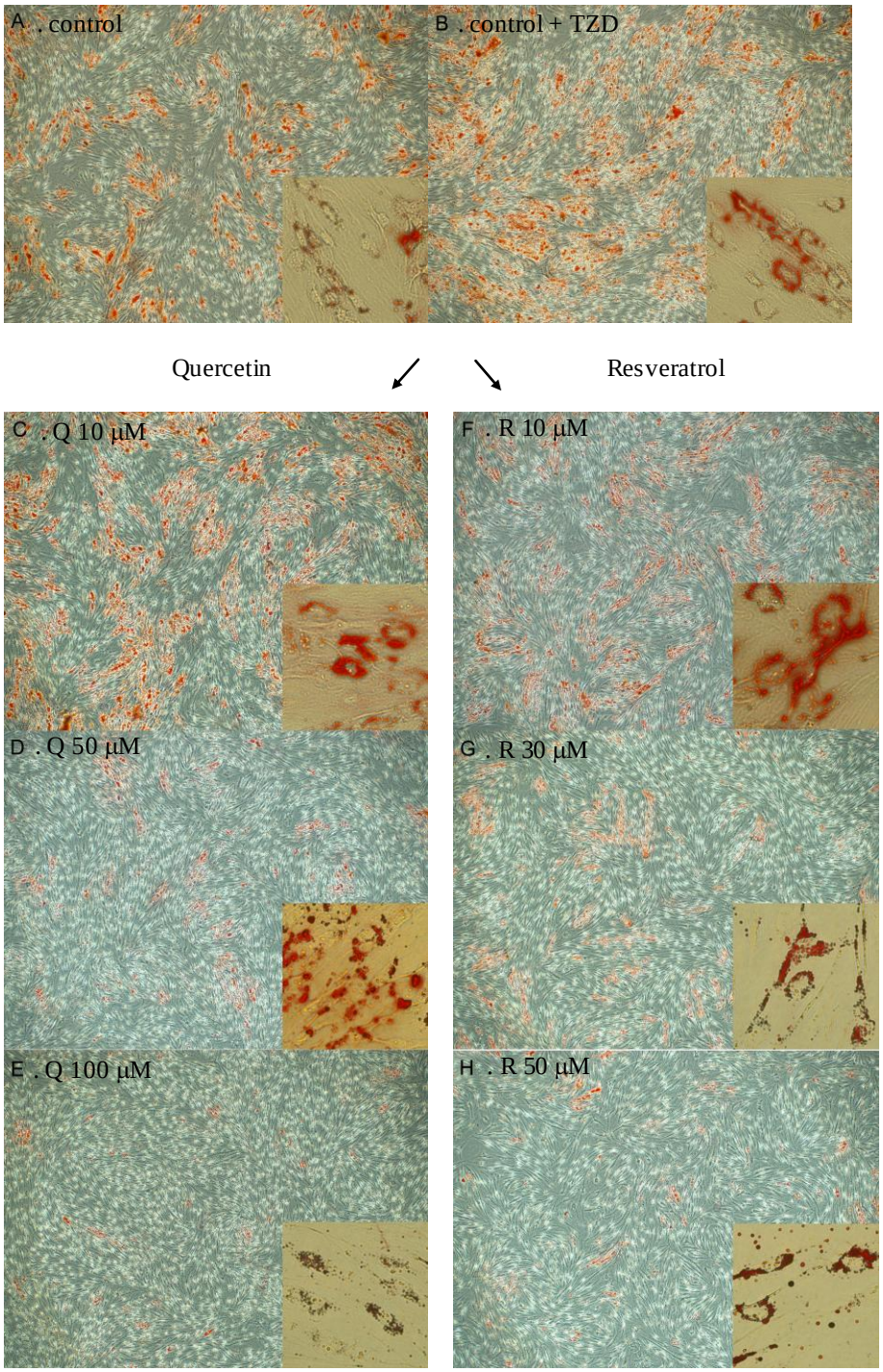
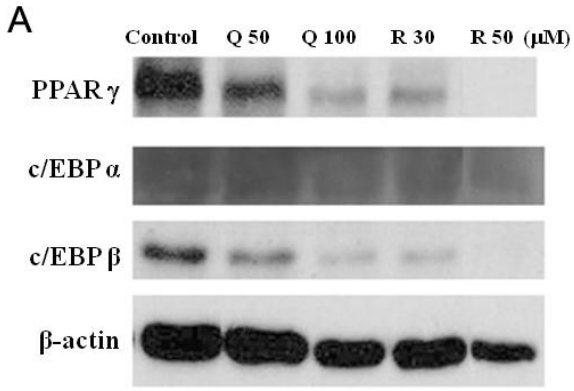
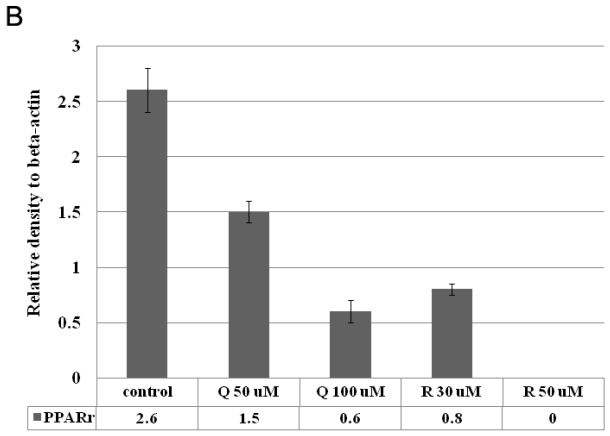

A . control
B . control + TZD
Quercetin
Resveratrol
C . Q 10 μM
D . Q 50 μM
E . Q 100 μM
F . R 10 μM
G . R 30 μM
H . R 50 μM

Figure 17. Microscopic examination of oil red O stained cultures exposed to quercetin and resveratrol. Orbital fibroblasts from a single individual with Graves' ophthalmopathy were cultured for 10 days in control adipogenic medium without rosiglitazone (A) and containing rosiglitazone 10 μM (B), and were fixed with 10% formalin for 1 hour and then stained with oil red O to measure lipid accumulation. Quercetin (C: 10, D: 50, E: 100 μM) and resveratrol (F: 10, G: 30, H: 50 μM) with varying concentrations were added to the differentiation medium containing rosiglitazone for the entire 10 day differentiation period, and were examined by light microscopy after oil red O stain. TZD = Thiazolidinediones (X40, X400: small box).

Western blot analysis was performed to investigate whether various concentrations of quercetin and resveratrol (10, 50, and 100 μM) affect the expression of adipogenic transcription factors such as PPARγ , C/EBPα and C/EBPβ. PPARγ and C/EBPβ proteins were highly expressed in control differentiated fibroblasts without treatment of quercetin and resveratrol. The increases in PPARγ and C/EBPβ were markedly attenuated dose dependently by quercetin and resveratrol as shown in Figure 18.


A
Control
Q 50
Q 100
R 30
R 50 (μM)
PPAR γ
c/EBP α
c/EBP β
β-actin


B
Relative density to beta-actin
3
2.5
2
1.5
1
0.5
0
control
Q 50 uM
Q 100 uM
R 30 uM
R 50 uM
PPARr
2.6
1.5
0.6
0.8
0

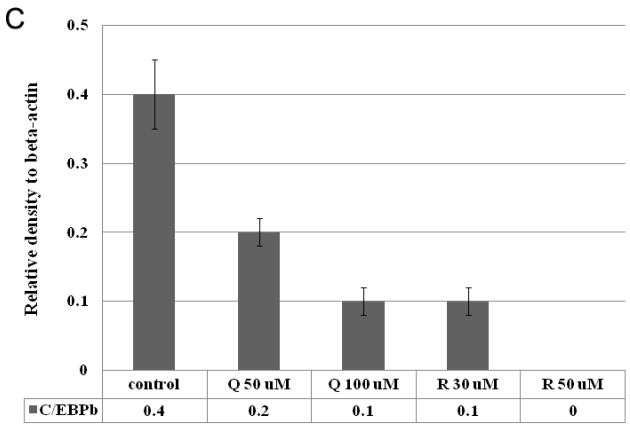

C
Relative density to beta-actin
0.5
0.4
0.3
0.2
0.1
0
control
Q 50 uM
Q 100 uM
R 30 uM
R 50 uM
C/EBPb
0.4
0.2
0.1
0.1
0

Figure 18. Effect of quercetin and resveratrol on the expression of adipogenic transcriptional regulators of differentiated orbital fibroblasts from Graves' ophthalmopathy patients. Various doses of quercetin (50, 100 μM) and resveratrol (30, 50 μM) were co-applied with differentiation medium including rosiglitazone 10 μM for 10 days. After differentiation was completed, cell lysates were prepared and subjected to western blot for PPARγ and C/EBP α, β protein. Representative figure of western blot analysis is shown in (A). Significant dose dependent-inhibitions of PPARγ (B) and C/EBP β (C) proteins by both quercetin and resveratrol were noted. Relative densities of PPARγ, C/EBP β protein contents (%) normalized by reprobing with anti-β-actin Abs were quantified with a densitometer. Data in the columns represent the mean ± SD of three experiments.

6. Quercetin's effect on accumulation of lipid droplet and cell viability in differentiating fibroblasts treated with IL-1β or H_2O_2

Our results indicate that resveratrol induce a dose dependent cytotoxicity and inhibit proliferation both in preadipocyte fibroblasts and differentiating fibroblasts as shown in figure 4-7. As shown in figure 15-17, inhibition effect of resveratrol on the accumulation of lipid droplets both in prestained and stained cultures was more apparent than that of quercetin even at low dose (10 μM) treatment, which might be associated with suppressive action of resveratrol on proliferation.

In MTT analyses of quercetin treated cells, viability was maintained around 100%

in all treatment doses (10, 50, 100 μM) of quercetin at the 10 day of differentiation when grown under adipogenic medium without rosiglitazone. However, treatment of 10-100 μM quercetin suppressed viability significantly when cultured in adipogenic medium containing rosiglitazone. The effect of quercetin on cell viability seems to be dependent on culture condition. Quercetin decreased adipocyte numbers and clearly suppressed lipid droplet accumulation in differentiating orbital fibroblasts with minimal cytotoxicity. Author further evaluated the quercetin's effect on adipocyte differentiation depending on different cell condition. The condition was determined to be inflammatory by IL-1β treatment and to be stressed by oxidants, H_2O_2 in the early period of differentiation.

When preadipocyte orbital fibroblasts were cultured under adipogenic condition, exposed to exogenous IL-1β (10ng/ml) for the first 3 days, adipogenesis was enhanced as shown in figure 19 and 20. More cells became round and accumulated lipid droplets. Combination of TZD and IL-1β further stimulated adipogenesis compared with adipogenic condition containing IL-1β only (Figure 19B, 20B). A dose-dependent inhibition of adipogenesis by quercetin in cultures grown under adipogenic medium including IL-1β and TZD was visible as shown in figure 19 C,D and 20 C,D. The remained preadipocyte fibroblasts preserved their fibroblast-like morphology (figure 20).

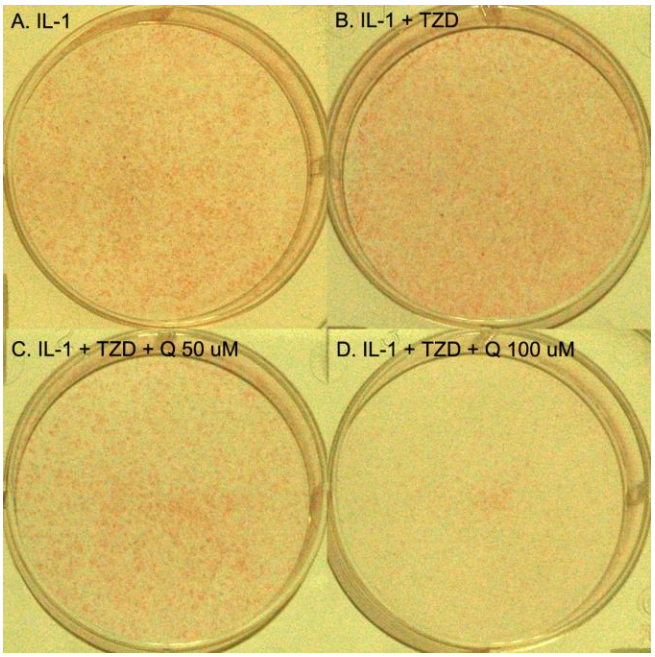


Figure 19. The effects of quercetin on adipocyte differentiation in cells exposed to adipogenic medium including IL-1β and quercetin. Orbital fibroblasts from a Graves' ophthalmopathy subject were cultured under adipogenic condition, exposed to exogenous IL-1β (10ng/ml) for the first 3 days (A) and also grown under media containing TZD (B –D) in orbital fibroblast from a patient with Graves' ophthalmopathy. The quercetin 50, 100 μM was co-applied (C, D) in adipogenic medium to demonstrate the suppressive effect of quercetin on adipogenesis in inflammatory condition. Cells were fixed with 10% formalin for 1 hour and then stained with oil red O to measure lipid accumulation. TZD = Thiazolidinediones

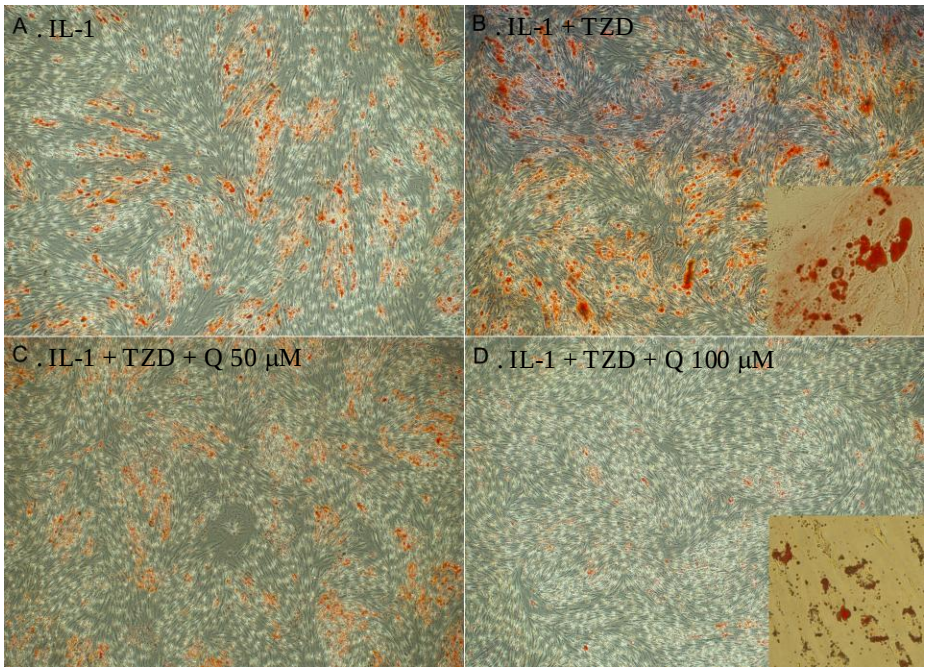


Figure 20. Microscopic results of oil red O stain showing suppressive effect of quercetin on adipogenesis in differentiating cells grown under adipogenic medium including IL-1β. Orbital fibroblasts from a Graves' ophthalmopathy subject were cultured under the adipogenic condition described in figure 19 (A: exposed to exogenous IL-1β 10ng/ml for the first 3 days only, B: IL-1β + rosiglitazone 10 μM, C: IL-1β + rosiglitazone + quercetin 50 μM, D: IL-1β + rosiglitazone + quercetin 100 μM, TZD = Thiazolidinediones).

H_2O_2 100 μM was exposed to differentiating orbital fibroblasts from a patient with Graves' ophthalmopathy for the first 3 days only, and the quercetin was co-applied in the adipogenic medium during 10 days of differentiation (Figure 21). As shown in figure 21, H_2O_2 100 μM treatment in early differentiation induced significant cell death. Interestingly, however, in quercetin treated cells, not only accumulation of lipid droplets decreased significantly in numbers, but also a considerable number of orbital fibroblasts preserved their normal fibroblastic morphology. We could observe a protective effect of quercetin on cell viability from the oxidative stress by H_2O_2 during adipocyte differentiation.

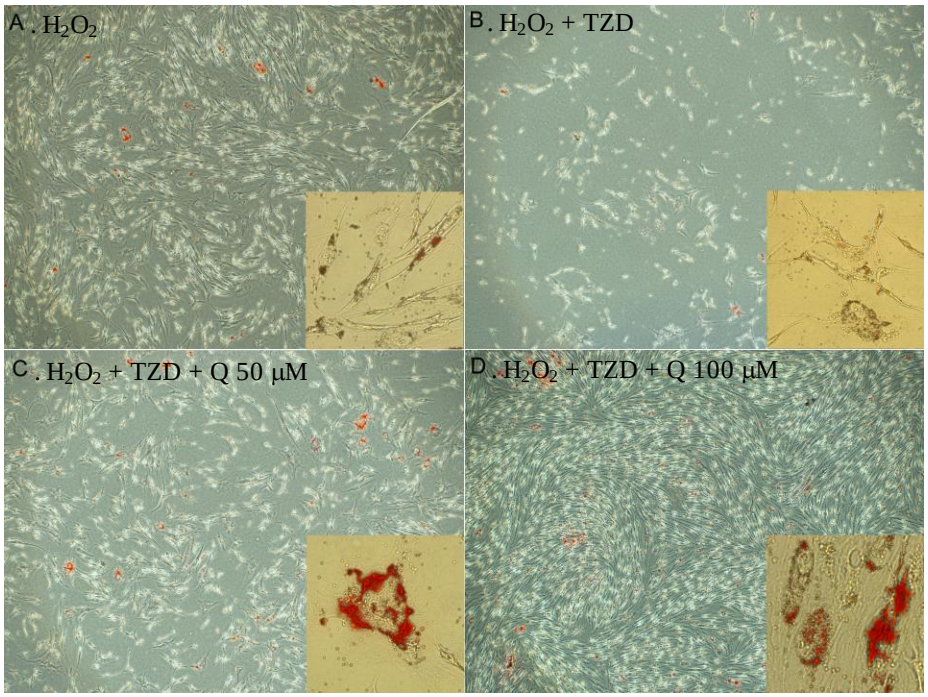
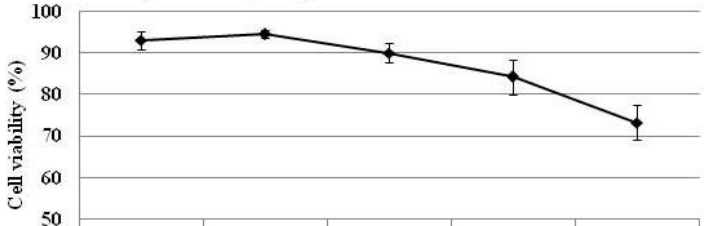


Figure 21. Microscopic results of oil red O stain showing protective effects of quercetin on cell viability in differentiating orbital fibroblasts exposed to H_2O_2. H_2O_2 100 μM was included in adipogenic medium for the first 3 days of differentiation (A-D). Rosiglitazone 10 μM was included in adipogenic medium in cultures (B-D), and quercetin 50 μM (C), 100 μM (D) was co-applied in adipogenic medium. Cells were fixed with 10% formalin for 1 hour and then stained with oil red O to measure lipid accumulation. TZD = Thiazolidinediones

To demonstrate the effect of quercetin on viability of fully differentiated orbital fibroblasts, MTT analyses were performed after quercetin treatment in fully differentiated cells in various conditions, not in the process of adipocyte differentiation (Figure 22). Varying concentrations of quercetin (1-100 μM) were exposed for 48 hours in cells from a Graves' ophthalmopathy subject at day 10 of adipocyte differentiation (Figure 22A). In addition, quercetin 100 μM was treated for 48 hours in fully differentiated cells which were exposed to varying concentrations of IL-1β (10, 100 ng/ml) (Figure 22B) and H_2O_2 (100, 500, 1000 μM) (Figure 22 C) for the first 3 days of adipogenesis.

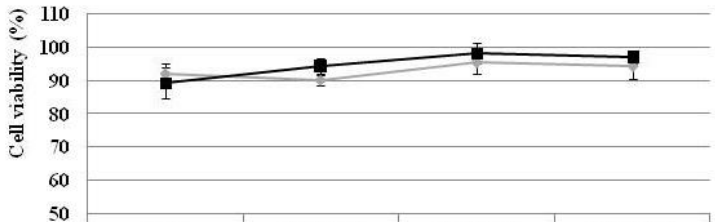
As shown in figure 22 A, quercetin decreased viability of fully differentiated fibroblasts dose dependently, which were similar to the MTT results in cells cultured in adipogenic medium containing quercetin. In contrast, cell viability was preserved with the treatment quercetin in cells exposed to IL-1β (10 ng/ml, 100 ng/ml both), and the quercetin did not decrease cell viability in these conditions (Figure 22 B). In other words, the inhibition effect of quercetin on adipogenesis in an *in vitro* inflammatory condition by IL-1β does not seem to be related to cytotoxicity. In concordance with microscopic results, H_2O_2 induced cell death dose dependently, and the application of quercetin increased viability significantly in a dose dependent manner (Figure 22 C). Quercetin treatment during and after adipogenesis both attenuated H_2O_2-induced cytotoxicity, as demonstrated in microscopic examination and MTT analyses.

A. Quercetin (1-100 uM, 48 h) treatment



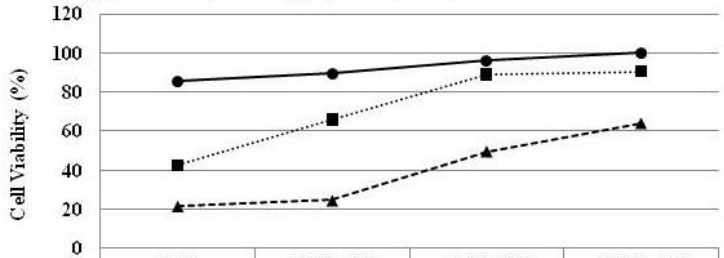
	Q 1 uM	Q 5 uM	Q 10 uM	Q 50 uM	Q 100 uM
%	93.03	94.50	90.06	84.2	73.28

B. IL-1 β (first 3 days of adipogenesis) + Quercetin



	Q (-)	Q 10 uM	Q 50 uM	Q 100 uM
IL-1 beta 10 ng/ml	92.04	90.3	95.49	94.38
IL-1 beta 100 ng/ml	89.51	94.3	98.3	97.03

C. H_2O_2 (first 3 days of adipogenesis) + Quercetin



	Q (-)	Q 10 uM	Q 50 uM	Q 100 uM
H2O2 100 uM	85.63	89.51	96.24	100
H2O2 500 uM	42.44	65.72	88.72	90.3
H2O2 1000 uM	21.54	24.54	49.56	64.01

Figure 22. MTT analyses after the treatment of quercetin in fully differentiated orbital fibroblasts in different adipogenic condition. Orbital fibroblasts from a single individual with Graves' ophthalmopathy were cultured for 10 days in control adipocyte differentiation medium including rosiglitazone, as described in Materials and Methods. After full adipocyte differentiation, quercetin with various concentrations (1-100 μM) was treated for 48 hours (A). To study the effect the quercetin on viability in cells treated with proinflammatory cytokine and oxidant, quercetin (10, 50, 100 μM) was treated for 48 hours in adipocyte fibroblasts which were stimulated with IL-1β (10, 100 ng/ml) (B) and H_2O_2 (100, 500, 1000 μM) (C) only for the first 3 days of differentiation. Data are average of three independent experiments ± SD.

IV. DISCUSSION

Graves' ophthalmopathy is the most frequent extrathyroidal manifestation of autoimmune hyperthyroidism. Many clinical signs and symptoms of Graves' ophthalmopathy arise from the enlargement of soft tissues in the orbit, resulting in increased pressure within the bony orbit.[31,32] Enlargement of both extraocular muscle and adipose tissue is found in some patients, with a predominance of one or the other in some.[32] Current evidence points to orbital fibroblasts as the target cells, and the thyrotropin receptor in orbital fibroblasts is the primary autoantigen in Graves' ophthalmopathy. Orbital fibroblasts particularly exhibit robust response to many proinflammatory mediators.[33] They critically orchestrate the recruitment of immunocompetent cells and thus initiation of tissue remodeling.[33] These orbital fibroblasts secrete large amount of hyaluronan in response to various cytokines,[33] and a subgroup of orbital fibroblasts can differentiate into mature adipocytes,[4] that have increased expression of thyrotropin receptor.[34, 35]

Graves' ophthalmopathy is a disfiguring and often incapacitating disease which is difficult to treat. Glucocorticoids have been the mainstay in the treatment of Graves' ophthalmopathy for a long time in spite of several frustrating complications. Orbital radiotherapy is an alternative treatment of Graves' ophthalmopathy with few adverse events. However, both treatments have a limited role when Graves' ophthalmopathy is not in active inflammation, especially with mild severity. Other immunosuppressants such as cyclosporine and methotrexate are inferior to glucocorticoids in reducing inflammation and halting the progression into severe course of Graves' ophthalmopathy. There is a need to improve the efficacy and tolerability of

immunosuppressive treatment of Graves' ophthalmopathy. Otherwise, new treatments with minimal adverse effects are eagerly awaited, which are effective in the major known pathogenesis of Graves' ophthalmopathy, including inflammation, hyaluronan production, and adipogenesis. In this study, we wished to determine whether quercetin and resveratrol could modify the proinflammatory cytokines expression, glycosaminoglycan synthesis and adipogenesis in primary cultured orbital fibroblasts from patients with Graves' ophthalmopathy. We have found these two chemicals showed somewhat different patterns of therapeutic effects of Graves' ophthalmopathy in the *in vitro* experiments.

Quercetin, a flavonol found in fruit and vegetables, has unique biological properties that include anti-inflammatory activity.[22] In the present investigation, we have observed that quercetin showed a treatment dose- and time-dependent inhibition of IL-1β induced proinflammatory cytokines expression in mRNA levels. It is reported that quercetin suppressed the production of TNFα and nitric oxide by macrophages, microglial cells and mast cells stimulated with lipopolysaccharide.[36, 37] Different mechanisms are involved in the anti-inflammatory action of quercetin in many cell systems, as it targets multiple intracellular signaling pathways such as signal transducer and activator of transcription 1 and NF-kB activations and mitogen-activated protein kinase family phosphorylatons.[38, 39]

Natural products have potential for inducing apoptosis, inhibiting adipocyte differentiation, and stimulating lipolysis in adipocytes. Various flavonoids including genistein, docosahexanoic acid, epigallocathechin gallate, quercetin and resveratrol

affect adipocytes during specific stages of development, resulting in either inhibition of adipogenesis or induction of apoptosis. Quercetin is reported to affect adipocytes during specific stages of development, resulting in either inhibition of adipogenesis or induction of apoptosis.[24, 40] It caused a dose- and time-dependent increase in lipolysis in rat adipocytes, which was reported to be potent phosphodiesterase inhibitors.[41] In this study, treatment of quercetin during adipogenesis of orbital fibroblasts from Graves' ophthalmopathy inhibited accumulation of lipid droplet and decreased number of round shape adipocytes in a dose-dependent manner in microscopic examination. The expressions of transcriptional regulators of adipogenesis, PPARγ and C/EBPβ proteins were also markedly attenuated by quercetin. In MTT analyses, quercetin did not reduce cell viability in differentiating cells in the absence of PPARγ agonist. However, quercetin decreased cell viability by 20-30% similarly both in differentiating and fully differentiated orbital fibroblasts under adipogenic condition containing PPARγ agonist. In microscopic examination, quercetin treatment preserved viable fibroblastic morphology of remained preadipocyte fibroblasts which were not differentiated. Orbital fibroblasts from Graves' ophthalmopathy might have different sensitivities regarding cytotoxicity of phytochemicals depending on cell status, such as different stage of adipocyte life cycles, inflammatory or oxidative stressed condition.

Quercetin significantly inhibited adipogenesis without induction of apoptosis in cells exposed to IL-1β. Stimulation with IL-1β *in vitro* can mimic orbital inflammation in Graves' ophthalmopathy. Consistent with a previous reports,[42,43] we found IL-1β had a stimulatory effect on adipogenesis in orbital fibroblasts, which may have important

clinical implications. IL-1β promoted all three pathological aspects of Graves' ophthalmopathy, those are inflammation, hyaluronan production and adipogenesis in our investigations. IL-1β might present an attractive therapeutic target in Graves' Ophthalmopathy. Quercetin not only suppressed IL-1β induced proinflammatory cytokines, hyaluronan production, but also inhibited adipocyte differentiation enhanced by IL-1β. The cytotoxic effects of quercetin in cells exposed to IL-1β during and after adipogenesis were minimal in microscopic examination and MTT analyses. Quercetin is believed to be a strong inhibitor of adipogenesis of orbital fibroblasts which target different stages of adipocyte life cycle, however not a strong inducer of apoptosis.

The particularly interesting result was that the quercetin protected cells from H_2O_2 induced cytotoxicity. Stimulation with H_2O_2 *in vitro* can mimic condition of cigarette smoking *in vivo*, which is well known as the strongest risk factor for developing severe Graves' ophthalmopathy. We could also observe that the differentiating orbital fibroblasts were highly vulnerable to H_2O_2, the oxidant. However, treatment of quercetin during and after adipogenesis significantly attenuated H_2O_2-induced cytotoxicity in orbital fibroblasts in microscopic results and MTT analyses. Cell viability recovered with the treatment of quercetin in a dose-dependent manner. This protective effect from acute stress may be associated with antioxidant property of quercetin, which is reported to show structural features that have been related to the antioxidant potency of flavonoids and also shows protection in different models of oxidative death.[18, 44-46] A novel mechanism for quercetin-induced cytoprotection has been described involving the sterol regulatory element-binding protein-2

(SREBP-2)-mediated sterol synthesis that decreases lipid peroxidation by maintaining membrane integrity in the presence of oxidative stress.[47] It is required to further investigate a cytoprotective mechanism of quercetin in orbital fibroblasts.

Resveratrol, a naturally-occurring molecule known as a phytoalexin, is synthesized by plants in response to attacks by fungi, bacteria, or other injurious substances.[36, 48,49] Resveratrol has positive effects on metabolism and can increase the lifespan of various organisms.[49] Its effects arise from its capacity to interact with multiple molecular targets involved in diverse intracellular pathways. Most well known is the ability of resveratrol to activate sirtuins, a class of NAD(+)-dependent deacetylases that affect multiple transcription factors and other protein targets.[49] In our study, resveratrol had a significant suppressive effect on IL-1β induced proinflammatory cytokine expressions in mRNA levels in orbital fibroblasts. The inhibitions of IL-1β induced IL-8 and COX-2 mRNA expression were dose dependent. In previous experiments in other cell systems and *in vivo*, resveratrol dampened inflammation in arthritis and immune responsiveness in autoimmune disease such as rheumatoid arthritis.[49, 50] It could also down regulate inflammatory biomarkers such as TNFα, COX-2, inducible nitric oxide synthase, C-reactive protein, interferon-γ and several interleukins.[49]

Resveratrol is reported to decrease fat mass by inhibiting adipogenesis and induce apoptosis by affecting expression of genes that modulate mitochondrial function.[29] It has been shown to inhibit adipogenesis by repressing PPARγ activity with activation of Sirt 1 in 3T3-L1 adipocytes.[51] In our study, as expected, resveratrol suppressed

accumulation of lipid droplet in differentiating cells dose dependently. It also blocked the expression of PPARγ and C/EBPβ proteins in a dose-dependent manner. The inhibition effects were even stronger than those of quercetin at low dose treatment. The difference of the effect of resveratrol from the quercetin was the inhibition of proliferation as shown in MTT and BrdU analyses. Cell viability decreased almost by 50% after treatment of resveratrol 100 μM both in preadipocyte fibroblasts and differentiating fibroblasts regardless of addition of PPARγ agonist. The mechanisms by which resveratrol induces apoptosis may be mediated through any of numerous mechanisms that involve activation of mitochondria and of death caspases. In 3T3-L1 adipocytes, treatment of resveratrol in maturing cells inhibited lipid accumulation and downregulated the expression of PPARγ, C/EBP, SREBP-1c and lipoprotein lipase, and also increased apoptosis in mature adipocytes dose dependently.[29] Recently, both quercetin and resveratrol inhibited lipid accumulation and induced apoptosis in early– and mid– phases maturing and lipid-filled mature primary human adipocytes.[24] The inhibitory effects on adipogenesis and apoptosis were greater when treated in combination including genistein.[24] In this study, resveratrol seems be a strong inhibitor of adipogenesis and also an inducer of apoptosis of maturing and matured orbital fibroblasts.

Thiazolidinediones (TZD), which are also called glitazones, are commonly used as oral hypoglycemic agents in the treatment of type 2 diabetes mellitus. These agents are shown to be potent agonists of the nuclear hormone receptor, PPARγ which plays a dominant role in adipocyte differentiation. A primary cause of proptosis in Graves' ophthalmopathy is the expansion of adipose tissue volume in the orbit.[52] In primary

cultured orbital fibroblasts, the activation of PPARγ by agonists was reported to stimulate functional TSH receptor expression and to induce recruitment and differentiation of orbital fibroblasts into mature lipid-laden adipocytes.[53-55] Several case studies found that Graves' ophthalmopathy was exacerbated by the use of TZD and stabilized after discontinuation.[56-59] These studies suggest that inhibition of adipogenic pathway through the use of PPARγ inhibitor might be a potential therapy for Graves' ophthalmopathy. In our study, orbital preadipocyte fibroblasts derived from Graves' ophthalmopathy patients were treated with flavonoid quercetin and resveratrol at various concentrations during the differentiation period. The differentiation media contained rosiglitazone, one of the members of TZD to induce strong adipogenic differentiation. Decrease in the lipid accumulation by oil red O stain was accompanied by attenuated expression of this adipogenic transcription factor, PPARγ and C/EBPβ by treatment of quercetin and resveratrol in a dose-dependent manner. PPARγ, C/EBP transcription factors expressed in distinct phases of adipogenesis have shown to play important roles. There is a positive feedback loop between PPARγ and C/EBPβ during the terminal stages of adipogenesis.[60] Our data suggest that quercetin and resveratrol exert anti-adipogenic effects by suppressing these adipogenic transcription factors.

Graves' ophthalmopathy is characterized by an inflammation of retrobulbar tissues, leading to accumulation of hydrophilic glycosaminoglycan, which attracts water into surrounding tissues and thereby increase volume of orbital connective tissue and extraocular muscles.[31, 61] Orbital fibroblasts *in vitro* respond to various mediators of inflammation, such as IL-1β by producing excessive amounts of hyaluronan, which is

a major glycosaminoglycan in orbital tissues of Graves' ophthalmopathy patients.[61,62] In this study, both IL-1β and TNF-α increased production of hyaluronan as previously reported, [61,62] and the pretreatment of quercetin suppressed the level of hyaluronan in orbital fibroblasts from both normal and Graves' ophthalmopathy individuals. We could not found any previous reports regarding the effect of quercetin on the production of glycosaminoglycan in any cell systems. Contrary to our expectation, resveratrol could not attenuate IL-1β or TNFα induced hyaluronan production.

Quercetin is reported to have the capacity to reduce acute, chronic, and subclinical inflammatory processes, the latter being associated with lifestyle diseases such as obesity and diabetes.[46] It is now available in high grade purified form, and clinical phase I-III studies can be readily done in the near fugure.[46] Resveratrol, the most potent natural sirtuin activator, together with the much more potent synthetic sirtuin activators, have considerable potential in the prevention and treatment of several common conditions of aging. The beneficial effects are supported by detailed findings, at the molecular and cellular level, of the specific pathways and molecules affected. However, there are many questions regarding flavoniods that remain to be investigated. Whether flavoniods act as pro- or anti-inflamamtory and pro- or anti-oxidants may depend on the differences in flavonoid concentration, cell type, and/or culture conditions. It is unknown whether these flavonoids contribute to the clinical benefits seen in the epidemiologic studies. However, we believe the results in this study are noteworthy as specific phytochemicals could be used as lead molecules to develop a new generation of drugs for the treatment of Graves' ophthalmopathy. The flavonoids could be safer and more natural with minimal side effects than high

dose glucocorticoids. Further research and more clinical studies are necessary in order to ensure the safety of these and for ascertaining the optimum doses for prevention and treatment of Graves' ophthalmopathy, bearing in mind that these molecules seem to have tissue and concentration-specific effects.

V. CONCLUSION

Flavonoids, quercetin and resveratrol possessed significant anti-inflammatory and anti-adipogenic effects in primary cultured orbital fibroblasts from Graves' ophthalmopathy. Quercetin also inhibited hyaluronan production induced by proinflammatory mediators. Our study results provided a basis for further study on the potential use of quercetin and resveratrol for the treatment of Graves' ophthalmopathy.

REFERENCES

1. Garrity JA, Bahn RS. Pathogenesis of graves ophthalmopathy: implications for prediction, prevention, and treatment. Am J Ophthalmol 2006 Jul;142(1):147-53.

2. Kuriyan AE, Phipps RP, Feldon SE. The eye and thyroid disease. Curr Opin Ophthalmol 2008 Nov;19(6):499-506.

3. Lehmann GM, Feldon SE, Smith TJ, Phipps RP. Immune mechanisms in thyroid eye disease. Thyroid 2008 Sep;18(9):959-65.

4. Sorisky A, Pardasani D, Gagnon A, Smith TJ. Evidence of adipocyte differentiation in human orbital fibroblasts in primary culture. J Clin Endocrinol Metab 1996 Sep;81(9):3428-31.

5. Crisp M, Starkey KJ, Lane C, Ham J, Ludgate M. Adipogenesis in thyroid eye disease. Invest Ophthalmol Vis Sci 2000 Oct;41(11):3249-55.

6. Bartley GB, Fatourechi V, Kadrmas EF, Jacobsen SJ, Ilstrup DM, Garrity JA, et al. Long-term follow-up of Graves ophthalmopathy in an incidence cohort. Ophthalmology 1996 Jun;103(6):958-62.

7. Bartalena L, Pinchera A, Marcocci C. Management of Graves' ophthalmopathy: reality and perspectives. Endocr Rev 2000 Apr;21(2):168-99.

8. Salvi M, Vannucchi G, Campi I, Curro N, Dazzi D, Simonetta S, et al. Treatment of Graves' disease and associated ophthalmopathy with the anti-CD20 monoclonal antibody rituximab: an open study. Eur J Endocrinol 2007 Jan;156(1):33-40.

9. Kung AW, Michon J, Tai KS, Chan FL. The effect of somatostatin versus corticosteroid in the treatment of Graves' ophthalmopathy. Thyroid 1996 Oct;6(5):381-4.

10. Dickinson AJ, Vaidya B, Miller M, Coulthard A, Perros P, Baister E, et al. Double-blind, placebo-controlled trial of octreotide long-acting repeatable (LAR) in thyroid-associated ophthalmopathy. J Clin Endocrinol Metab 2004 Dec;89(12):5910-5.

11. Stan MN, Garrity JA, Bradley EA, Woog JJ, Bahn MM, Brennan MD, et al. Randomized, double-blind, placebo-controlled trial of long-acting release octreotide for treatment of Graves' ophthalmopathy. J Clin Endocrinol Metab 2006 Dec;91(12):4817-24.

12. Paridaens D, van den Bosch WA, van der Loos TL, Krenning EP, van Hagen PM. The effect of etanercept on Graves' ophthalmopathy: a pilot study. Eye (Lond) 2005 Dec;19(12):1286-9.

13. Durrani OM, Reuser TQ, Murray PI. Infliximab: a novel treatment for sight-threatening thyroid associated ophthalmopathy. Orbit 2005 Jun;24(2):117-9.

14. Pietta PG. Flavonoids as antioxidants. J Nat Prod 2000 Jul;63(7):1035-42.

15. Cotelle N. Role of flavonoids in oxidative stress. Curr Top Med Chem 2001 Dec;1(6):569-90.

16. Formica JV, Regelson W. Review of the biology of Quercetin and related bioflavonoids. Food Chem Toxicol 1995 Dec;33(12):1061-80.

17. Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA. Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 2001 Oct;74(4):418-25.

18. Perez-Vizcaino F, Duarte J, Andriantsitohaina R. Endothelial function and cardiovascular disease: effects of quercetin and wine polyphenols. Free Radic Res 2006 Oct;40(10):1054-65.

19. Ong CS, Tran E, Nguyen TT, Ong CK, Lee SK, Lee JJ, et al. Quercetin-induced growth inhibition and cell death in nasopharyngeal carcinoma cells are associated with increase in Bad and hypophosphorylated retinoblastoma expressions. Oncol Rep 2004 Mar;11(3):727-33.

20. Mertens-Talcott SU, Talcott ST, Percival SS. Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. J Nutr 2003 Aug;133(8):2669-74.

21. Kawada N, Seki S, Inoue M, Kuroki T. Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. Hepatology 1998 May;27(5):1265-74.

22. Comalada M, Camuesco D, Sierra S, Ballester I, Xaus J, Galvez J, et al. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. Eur J Immunol 2005 Feb;35(2):584-92.

23. Ying B, Yang T, Song X, Hu X, Fan H, Lu X, et al. Quercetin inhibits IL-1 beta-induced ICAM-1 expression in pulmonary epithelial cell line A549 through the MAPK pathways. Mol Biol Rep 2009 Sep;36(7):1825-32.

24. Park HJ, Yang JY, Ambati S, Della-Fera MA, Hausman DB, Rayalam S, et al. Combined effects of genistein, quercetin, and resveratrol in human and 3T3-L1 adipocytes. J Med Food 2008 Dec;11(4):773-83.

25. Ahn J, Lee H, Kim S, Park J, Ha T. The anti-obesity effect of quercetin is mediated by the AMPK and MAPK signaling pathways. Biochem Biophys Res Commun 2008 Sep 5;373(4):545-9.

26. Visentin V, Prevot D, Marti L, Carpene C. Inhibition of rat fat cell lipolysis

by monoamine oxidase and semicarbazide-sensitive amine oxidase substrates. Eur J Pharmacol 2003 Apr 18;466(3):235-43.

27. Donnelly LE, Newton R, Kennedy GE, Fenwick PS, Leung RH, Ito K, et al. Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms. Am J Physiol Lung Cell Mol Physiol 2004 Oct;287(4):L774-83.

28. ElAttar TM, Virji AS. Modulating effect of resveratrol and quercetin on oral cancer cell growth and proliferation. Anticancer Drugs 1999 Feb;10(2):187-93.

29. Rayalam S, Yang JY, Ambati S, Della-Fera MA, Baile CA. Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes. Phytother Res 2008 Oct;22(10):1367-71.

30. Green H, Kehinde O. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 1975 May;5(1):19-27.

31. Bahn RS, Heufelder AE. Pathogenesis of Graves' ophthalmopathy. N Engl J Med 1993 Nov 11;329(20):1468-75.

32. Bahn RS. Graves' ophthalmopathy. N Engl J Med Feb 25;362(8):726-38.

33. Smith TJ. Orbital fibroblasts exhibit a novel pattern of responses to proinflammatory cytokines: potential basis for the pathogenesis of thyroid-associated ophthalmopathy. Thyroid 2002 Mar;12(3):197-203.

34. Valyasevi RW, Erickson DZ, Harteneck DA, Dutton CM, Heufelder AE, Jyonouchi SC, et al. Differentiation of human orbital preadipocyte fibroblasts induces expression of functional thyrotropin receptor. J Clin Endocrinol Metab 1999 Jul;84(7):2557-62.

35. Starkey KJ, Janezic A, Jones G, Jordan N, Baker G, Ludgate M. Adipose

thyrotrophin receptor expression is elevated in Graves' and thyroid eye diseases ex vivo and indicates adipogenesis in progress in vivo. J Mol Endocrinol 2003 Jun;30(3):369-80.

36. Bureau G, Longpre F, Martinoli MG. Resveratrol and quercetin, two natural polyphenols, reduce apoptotic neuronal cell death induced by neuroinflammation. J Neurosci Res 2008 Feb 1;86(2):403-10.

37. Kumazawa Y, Kawaguchi K, Takimoto H. Immunomodulating effects of flavonoids on acute and chronic inflammatory responses caused by tumor necrosis factor alpha. Curr Pharm Des 2006;12(32):4271-9.

38. Hamalainen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediators Inflamm 2007;2007:45673.

39. Lee KW, Kang NJ, Heo YS, Rogozin EA, Pugliese A, Hwang MK, et al. Raf and MEK protein kinases are direct molecular targets for the chemopreventive effect of quercetin, a major flavonol in red wine. Cancer Res 2008 Feb 1;68(3):946-55.

40. Rayalam S, Della-Fera MA, Baile CA. Phytochemicals and regulation of the adipocyte life cycle. J Nutr Biochem 2008 Nov;19(11):717-26.

41. Kuppusamy UR, Das NP. Potentiation of beta-adrenoceptor agonist-mediated lipolysis by quercetin and fisetin in isolated rat adipocytes. Biochem Pharmacol 1994 Feb 9;47(3):521-9.

42. Cawood TJ, Moriarty P, O'Farrelly C, O'Shea D. Smoking and thyroid-associated ophthalmopathy: A novel explanation of the biological link. J Clin Endocrinol Metab 2007 Jan;92(1):59-64.

43. Cawood TJ, Moriarty P, O'Farrelly C, O'Shea D. The effects of tumour necrosis factor-alpha and interleukin1 on an in vitro model of thyroid-associated ophthalmopathy; contrasting effects on adipogenesis. Eur J Endocrinol 2006 Sep;155(3):395-403.

44. Loke WM, Proudfoot JM, McKinley AJ, Needs PW, Kroon PA, Hodgson JM, et al. Quercetin and its in vivo metabolites inhibit neutrophil-mediated low-density lipoprotein oxidation. J Agric Food Chem 2008 May 28;56(10):3609-15.

45. Huk I, Brovkovych V, Nanobash Vili J, Weigel G, Neumayer C, Partyka L, et al. Bioflavonoid quercetin scavenges superoxide and increases nitric oxide concentration in ischaemia-reperfusion injury: an experimental study. Br J Surg 1998 Aug;85(8):1080-5.

46. Bischoff SC. Quercetin: potentials in the prevention and therapy of disease. Curr Opin Clin Nutr Metab Care 2008 Nov;11(6):733-40.

47. Soundararajan R, Wishart AD, Rupasinghe HP, Arcellana-Panlilio M, Nelson CM, Mayne M, et al. Quercetin 3-glucoside protects neuroblastoma (SH-SY5Y) cells in vitro against oxidative damage by inducing sterol regulatory element-binding protein-2-mediated cholesterol biosynthesis. J Biol Chem 2008 Jan 25;283(4):2231-45.

48. Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G, Goldberg DM. The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. Clin

Chim Acta 1995 Mar 31;235(2):207-19.

49. Shakibaei M, Harikumar KB, Aggarwal BB. Resveratrol addiction: to die or not to die. Mol Nutr Food Res 2009 Jan;53(1):115-28.

50. Csaki C, Mobasheri A, Shakibaei M. Synergistic chondroprotective effects of curcumin and resveratrol in human articular chondrocytes: inhibition of IL-1beta-induced NF-kappaB-mediated inflammation and apoptosis. Arthritis Res Ther 2009;11(6):R165.

51. Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, et al. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature 2004 Jun 17;429(6993):771-6.

52. Kumar S, Coenen MJ, Scherer PE, Bahn RS. Evidence for enhanced adipogenesis in the orbits of patients with Graves' ophthalmopathy. J Clin Endocrinol Metab 2004 Feb;89(2):930-5.

53. Valyasevi RW, Harteneck DA, Dutton CM, Bahn RS. Stimulation of adipogenesis, peroxisome proliferator-activated receptor-gamma (PPARgamma), and thyrotropin receptor by PPARgamma agonist in human orbital preadipocyte fibroblasts. J Clin Endocrinol Metab 2002 May;87(5):2352-8.

54. Pistrosch F, Herbrig K, Oelschlaegel U, Richter S, Passauer J, Fischer S, et al. PPARgamma-agonist rosiglitazone increases number and migratory activity of cultured endothelial progenitor cells. Atherosclerosis 2005 Nov;183(1):163-7.

55. Mimura LY, Villares SM, Monteiro ML, Guazzelli IC, Bloise W. Peroxisome proliferator-activated receptor-gamma gene expression in orbital adipose/connective tissues is increased during the active stage of Graves' ophthalmopathy. Thyroid 2003 Sep;13(9):845-50.

56. Levin F, Kazim M, Smith TJ, Marcovici E. Rosiglitazone-induced proptosis. Arch Ophthalmol 2005 Jan;123(1):119-21.

57. Dorkhan M, Lantz M, Frid A, Groop L, Hallengren B. Treatment with a thiazolidinedione increases eye protrusion in a subgroup of patients with type 2 diabetes. Clin Endocrinol (Oxf) 2006 Jul;65(1):35-9.

58. Starkey K, Heufelder A, Baker G, Joba W, Evans M, Davies S, et al. Peroxisome proliferator-activated receptor-gamma in thyroid eye disease: contraindication for thiazolidinedione use? J Clin Endocrinol Metab 2003 Jan;88(1):55-9.

59. Lee S, Tsirbas A, Goldberg RA, McCann JD. Thiazolidinedione induced thyroid associated orbitopathy. BMC Ophthalmol 2007;7:8.

60. Zieleniak A, Wojcik M, Wozniak LA. Structure and physiological functions of the human peroxisome proliferator-activated receptor gamma. Arch Immunol Ther Exp (Warsz) 2008 Sep-Oct;56(5):331-45.

61. Kaback LA, Smith TJ. Expression of hyaluronan synthase messenger ribonucleic acids and their induction by interleukin-1beta in human orbital fibroblasts: potential insight into the molecular pathogenesis of thyroid-associated ophthalmopathy. J Clin Endocrinol Metab 1999 Nov;84(11):4079-84.

62. Smith TJ, Wang HS, Evans CH. Leukoregulin is a potent inducer of hyaluronan synthesis in cultured human orbital fibroblasts. Am J Physiol 1995 Feb;268(2 Pt 1):C382-8.

63. Hubbard GP, Wolffram S, Lovegrove JA, Gibbins JM. Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. J Thromb Haemost 2004

Dec;2(12):2138-45.

< ABSTRACT (IN KOREAN) >

갑상선안병증 in vitro 모델에서 quercetin과 resveratrol의 치료적 역할

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갑상선안병증은 아직까지 그 발생기전이 명확하지는 않으나, 전형적인 자가면역 염증질환으로, 안와섬유모세포의 급성 염증반응, hyaluronan 생성 및 지방분화의 증가로 인한 안와내 지방결체조직과 외안근의 팽창이 주요 기전으로 생각되고 있다. 고용량 스테로이드와 방사선치료가 활동성 염증기의 갑상선안병증에는 진행억제 효과가 있지만 비활동성, 만성염증 시기에는 효과가 없으며, 부작용으로 인해 사용에 한계가 있다. 본 연구자는 갑상선안병증을 일으키는 여러 병태생리학적 기전을 전반적으로 억제하면서 부작용이 적은 치료 방법을 찾아보고자 하였고 가장 유력한 물질로 Flavonoid, 이중 quercetin 과 resveratrol 을 선택하였다. 갑상선안병증 환자에서 안와감압술 시행후 버려지는 조직인 안와 지방결체조직으로부터 안와 섬유모세포를 일차 배양하고 비교실험을 위해 갑상선안병증이 없는 정상인을 대상으로 시행한 안와 수술 등을 통해 안와 지방결체조직을 얻어, 정상 안와섬유모세포도 일차 배양하였다. MTT, BrDU assay 와 annexin V-FITC 를 이용한 flow cytometry 실험을 통해 두 flavonoid 가 세포의 생존, 증식 및 세포사에 미치는 영향을 평가하였으며, 다양한 농도의 quercetin (0-100 μM)과 resveratrol (0-50 μM)을 안와섬유모세포에 전처치후, IL-1β 자극에 의해 증가된 ICAM-1, IL-6,

IL-8, COX-2 mRNA 표현이 억제되는지를 RT-PCR 을 이용하여 확인하였다. 또한, Hyaluronan ELISA를 이용하여 quercetin과 resveratrol의 전처치후, IL-1β 또는 TNF-α 자극에 의해 증가한 hyaluronan 농도가 감소하는지 측정하였다. 마지막으로, peroxisome proliferator activator gamma (PPARγ) agonist (Rosiglitazone)을 포함한 지방분화 유도 배양액에 quercetin 또는 resveratrol을 10일간 같이 처리한 세포에서 지방분화가 끝난 후, Oil red O 염색 결과 및 western blot 을 이용하여 PPARγ, C/EBP 단백질발현을 확인하였다.

MTT 분석결과, quercetin (0-100 μM), resveratrol (0-50 μM) 24 시간 처리시 quercetin 은 undifferentiated orbital fibroblast 의 생존에 악영향이 없었으나 resveratrol 은 농도 비례하여 세포사를 유도하였다. BrdU 분석결과, 저농도의 quercetin 은 약한 증식을 일으켰고 resveratrol은 농도에 비례하여 증식억제효과가 강하였다. Annexin V/FITC FACS 결과 quercetin 100 μM, resveratrol 50 μM까지 세포독성이 발생하지 않아, 최대 농도를 이와 같이 정하였다. 두 약물 모두 IL-1β (10ng/ml) 자극에 의한 ICAM-1, IL-6, IL-8 과 COX-2 mRNA 발현을 억제하였고 Hyaluronan 생성 억제 효과는 quercetin 에서만 관찰되었다. 두 약물 모두 지방분화를 유도하는 세포에 처리한 후 oil red O 염색한 결과, 농도의존적으로, lipid droplet 의 크기와 수가 감소하였고, 농도의존적으로 PPARγ 와 C/EBPβ 단백질 발현을 억제하였다. Quercetin 추가실험결과, IL-1β 10ng/ml 자극으로 지방분화를 증가시킨 세포에 quercetin을 농도별 처리한 결과, 세포 독성없이 농도의존적으로 지방분화를 감소시켰으며, H_2O_2 을 지방분화도중 혹은 지방분화 후 처리한 세포에서 quercetin 이 농도의존적으로 세포생존을 증가시킴을 관찰할 수 있었다.

Quercetin 과 resveratrol 은 *in vitro* 갑상선안병증 모델, 즉 일차 배양 안와 섬유모세포에서, 효과는 다르지만 치료약제로서의 가능성을 보여주었다. Quercetin 은 항염증효과, hyaluronan 생성 및 지방분화에 대한 전반적인 억제 효과를 나타냈으며, resveratrol 은 quercetin 과 마찬가지로 항염증효과 및 지방분화 억제 효과가 있었고, 농도의존적인

증식억제효과가 뚜렷하였다. 본 연구는 갑상선안병증의 새로운 치료제의 *in vitro* 에서의 가능성을 입증하는 연구로, 향후 동물 실험 또는 임상연구를 통해 약물의 유효성과 안전성 평가가 필요할 것이다.

핵심되는 말: 갑상선안병증, 안와섬유모세포, 염증, 지방분화, hyaluronan, quercetin, reveratrol