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Effect of Goji (*Lycium barbarum*) on Expression of Genes Related to Cell Survival

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ABSTRACT: This study investigated the interrelationship between *Lycium barbarum* (goji) and gene expression in mouse spleen. Oligomicroarray technology was employed to explore the comprehensive response of gene expression and to screen candidate marker genes in the spleens of mice fed a goji suspension. Goji was micronized by media milling and then used to evaluate the effect of size reduction. The average diameter of nano/submicrometer goji was about 100 nm, which exhibited no cytotoxicity to cell lines IEC-6 (rat normal small intestinal cell line) and Caco-2 (human colon adenocarcinoma cell line). It was found that three genes, TNF, Nfkb1, and Bcl-2, were up-regulated and two genes, APAF-1 and caspase-3, were down-regulated by goji. This phenomenon could be helpful for cytoprotection when cells undergo stress or damage that induces the apoptotic pathway. Size reduction into nano/submicrometer scale enhanced bioactivity.

KEYWORDS: *Lycium barbarum*, media milling, gene expression, oligomicroarray

INTRODUCTION

Goji (*Lycium barbarum* L.) is well-known in Asia for nourishing the liver and improving eyesight. In addition to colored components of zeaxanthin and carotene, polysaccharides and small molecules such as betaine, cerebroside, β-sitosterol, *p*-coumaric acid, and various vitamins are recognized as bioactive compounds in goji.[1] A high content of carotenoid (ranging from 0.03 to 0.5%) provides high provitamin A value[2] and antioxidative capacity. Zeaxanthin dipalmitate is the focus of current research for the prevention and treatment of age-related macular degeneration (AMD) and is a predominant carotenoid, comprising 31−56% of the total carotenoids in goji.[3] Zeaxanthin is an oxygenated carotenoid (xanthophyll) and is a structural isomer of lutein.[4] Both zeaxanthin and lutein are rich in fovea, or "yellow spot" of eyes, and are major compounds of macular pigment.[5] Extract of goji exhibits various functionalities, including a cytoprotective effect (by reducing caspase-3 activity),[6] an enhancing immune function,[7] a neuromodulation effect,[8] and an antioxidation activity.[9] Understanding the effect of goji on gene expression is essential in the exploration of the mechanisms of health benefits exhibited by goji.

Nutrigenomics integrates the knowledge of health, diet, and genomics, as well as molecular nutrition, to explore the role of nutrients in the metabolic pathway, in homeostatic control, and in the early phase of diet-related diseases.[10] Whole-genome gene expression profiles can provide new insights into nutrient−gene interactions and diet-related mechanisms underlying alterations in gene expression and functional states of the cell.[11] Microarray technology provides a more comprehensive, unbiased knowledge of all gene networks, including members of gene families, ligands, receptors, and transcription factors.[12] Additionally, microarray analysis allows for the discovery of new genes and/or pathways previously not known.[13] Green tea extracts and fructooligosaccharide are two examples of food components affecting gene expression as determined by microarray analysis, further confirmed by RT-PCR.[14,15]

Reducing size to a nano/submicrometer scale is believed to enhance the bioavailability of nutrients. Liao et al.[16] reported that nano/submicronization enhances the transport and absorption of lignan glycosides by Caco-2 cells. Size reduction of cellulose also enhances the rate and yield of enzymatic hydrolysis.[17] One economical way to reduce size is to employ a media mill, one of various types derived from stirred-ball mills.[18] Date and Patravale[19] pointed out that media-milling is a popular method to prepare nanoparticles in the drug industry. Few papers in the literature are concerned with the application of media-milling in food. The purpose of media-milling is to prepare hydrophobic materials at a nano/submicrometer scale, suspended in water and having a reasonable shelf life. Understanding the interactions between nano/submicrometer particles and genes would be helpful for developing health foods and for applying nanotechnology in food. However, possible adverse effects of nano/submicronization also need to be examined. The objectives of this study are to understand the effect of goji on gene expression and to explore potential benefits of nano/submicronization on the bioactivity of goji.

MATERIALS AND METHODS

Samples. Dry goji (*L. barbarum*) was purchased from the Tung-Jen Chinese herbal medicine store (Taipei, Taiwan) and stored in a desiccator at 25 °C until use. The contents of heavy metals (including

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Pb, Cd, Hg, and As) were determined using an ICP-OES (Perkin-Elmer AAnalyst 200 fitted with a flow injection system, FIAS 400) with a detection limit of 0.32 μg/L. The results showed that there were no detectable heavy metals in samples.

Media-Milled Goji Preparation. Nano/submicrometer goji particles were prepared using a media mill. Dried goji (10 g) was mixed with distilled water (500 mL) and minced in a high-speed blender (PT 3100, Kinematica, Lucerne, Switzerland) at 20000 rpm for 10 min to obtain blended goji suspension. The blended sample was further processed by a media mill (Minipur, Netzsch-Feinmahltechnik GmbH, Germany) with a driving motor of 0.94 kW to prepare goji suspension in nano/submicrometer scale, which was designated media-milled goji suspension. Media (0.8 mm, yttria-stabilized tetragonal zirconia, YTZ) at 70% v/v filling ratio were placed in the milling chamber (200 mL). The blended sample (400 mL) was loaded into a jacket-cooling tank and then fed at a flow rate of 600 mL/min into the milling chamber by a circulation pump. During milling, particles smaller than the gap (0.15 mm) of the media separator were brought out into the stirred tank for cooling by the circulation system. The temperature of the suspension was thus maintained below 16 °C during milling. The agitation speed was set at 3000 rpm, and the milling was conducted for 90 min.

Particle Size Distribution. Particle size distribution of sample was determined by using a laser diffraction particle size analyzer (LS 230, Beckman Coulter, Fullerton, CA) with a detecting range of 0.04−2000 μm. The instrument was calibrated with deionized water. All of the samples were diluted 2-fold, subjected to mild stirring, and then degassed by sonication (Branson 3510R-DTH, Branson Ultrasonic Corp., Danbury, CT) for 5 min at 100 W and 42 kHz. Average diameters (in volume and number) of particles were obtained using software with LS 230 (Beckman Coulter version 3.29). All of the measurements were done in triplicate and the average data reported.

Morphology. Morphology was examined by scanning electron microscopy (SEM) (Hitachi S-800, Hitachi Co. Ltd., Tokyo, Japan) and transmission electron microscopy (TEM) (JEM1230, JEOL Co. Ltd., Tokyo, Japan). Critical-point drying was employed to prepare samples for SEM observation. Goji suspension (blended or media-milled, 2 mL) was mixed with 18 mL of 75% ethanol and then was evaporated to 2 mL at vacuum condition. The replacement process was repeated twice using 95% ethanol. Final replacement was conducted using 99% ethanol, and solids were suspended in 2 mL of 99% ethanol, which was further dried using liquid CO_2 in a critical-point drying apparatus (Hitachi HCP-2, Hitachi Co. Ltd., Tokyo, Japan) at 31.1 °C and 73.9 bar. After being dried, the sample was attached on an SEM stub using double-backed cellophane tape. The stub and sample were coated with gold−palladium and were examined and photographed at 20 kV. For TEM, one drop of the sample solution was deposited on a carbon-coated microscope grid (200 mesh copper specimen grid, Agar Scientific Ltd., Essex, U.K.) and dried by natural air circulation at 25 °C. TEM observation was conducted with an acceleration voltage of 75 kV and 50000 magnifications.

Composition Analysis. The contents of crude protein, crude fat, crude ash, and moisture were measured using the methods of the Association of Official Analytical Chemists (AOAC).[20]

Analysis of Total Carotenoids, β-Carotene, and Zeaxanthin. The extraction and chromatographic fractionation of carotenoids in samples were conducted according to the method of Zhou et al.[21] Distilled water (2 mL) and 2 mL of 95% ethanol were added to 50 mg of lyophilized samples. The mixture was mixed with *n*-hexane (4-fold the volume of the mixture) and vortexed for 3 min to extract carotenoids. After the extraction and centrifugation at 10000*g* for 5 min, the organic phase was collected. The extraction was continued until the organic phase became colorless. The collected extract was dried using N_2 gas and designated dry extract. A spectrophotometric method was used to determine the total carotenoid content. Dry extract was dissolved in *n*-hexane (1 mL). Absorption at 450 nm, the maximum absorption of carotenoids zeaxanthin and β-carotene,[22] was determined using a spectrophotometer (Jasco V-530, UV−vis spectrophotometer, Tokyo, Japan). Total carotenoid content was calculated according to McBeth's formula[23] as

$$\frac{\text{mg carotenoids}}{100\text{ g sample}} = \frac{\text{OD} \times V \times 10^3}{E^{1\%}_{1\text{cm}} \times \text{weight of sample (g)}} \quad (1)$$

where optical density (OD) was determined by spectrophotometer, $E^{1\%}_{1\text{ cm}}$ was 1% of the average extinction coefficient value of carotenoids in *n*-hexane = 2500, and *V* was total volume of the solution (mL).

The dry extract was dissolved in mobile phase (16:84 v/v dioxane/*n*-hexane) for HPLC (Jasco UV-2075, UV−vis detector) analysis to determine the content of zeaxanthin and β-carotene. A C18 column (YMC-Pack ODS-AM, S-5 μM, 12 nm, YMC Co., Japan) eluted with an isocratic mobile phase of 16% dioxane in *n*-hexane at 1 mL/min was used to fractionate zeaxanthin and β-carotene. The injection volume was 20 μL. A UV detector was employed to determine absorption at 450 nm by the eluent.

Analysis of Crude Polysaccharides. Crude polysaccharides in samples were determined using the method of Gan et al.[24] with slight modification. Samples (including both blended and media-milled goji) were centrifuged at 10000*g* for 10 min. The supernatants were collected and designated cold water extract. The residues were further extracted with hot water (100 °C) for 2 h,[25] and the filtrate was collected and designated hot water extract. Both cold and hot water extracts were pooled together and then concentrated until the volume was $^1/_{20}$ of the original sample. Ethanol (95%) was used to precipitate polysaccharides at 4 °C overnight. The precipitate was dissolved in 100 °C distilled water and reprecipitated with 4 volumes of 95% ethanol to obtain crude polysaccharides. The content of crude polysaccharides was determined according to the phenol−sulfuric acid colorimetric method using glucose as a standard.[26]

Cell Viability of IEC-6 and Caco-2. In media-milled samples, >1% of particles were sized from 1 to 100 nm, in terms of number size distribution. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed to evaluate the effects of media-milled goji on viability of IEC-6 (rat small intestine epithelial cell line, ATCC CRL-1592) and Caco-2 cells (human colon adenocarcinoma cell line, ATCC HTB-37). Both cell lines were cocultured with various doses of samples for 24, 48, and 72 h. Cells without goji were utilized as a control group. Cell viability was evaluated by MTT assay and calculated as the percentage of the control group.[27] In brief, IEC-6 and Caco-2 cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified 95% air/5% CO_2 environment. Up to 80% confluent, cells were treated with trypsin and harvested by centrifugation (1000 rpm, 5 min). Cells were counted using trypan blue dye exclusion assay, and an equal number of cells (5×10^4 cells/mL) were transferred to a 96-well plate (Greiner Bio-One GmbH, Frickenhausen, Germany). Treated goji was filtered through a 0.45 μm membrane and then added to cell culture. The cells with an equivalent volume of DMEM medium were designated the control group. After incubation for 24, 48, and 72 h, MTT reagent (Sigma, St. Louis, MO) was added to each well. Optical density at 570 nm was determined using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA). The experiments were conducted in triplicate.

Statistical Analysis. Data were reported as the mean of experiments conducted in triplicate. One-way analysis of variance (ANOVA) was used to determine the significance of treatment using the Statistical Analysis System (SAS version 9.1; SAS Institute Inc., Cary, NC), followed by a Duncan's multiple-comparison test. Differences were considered as statistically significant when the *P* value was <0.05.

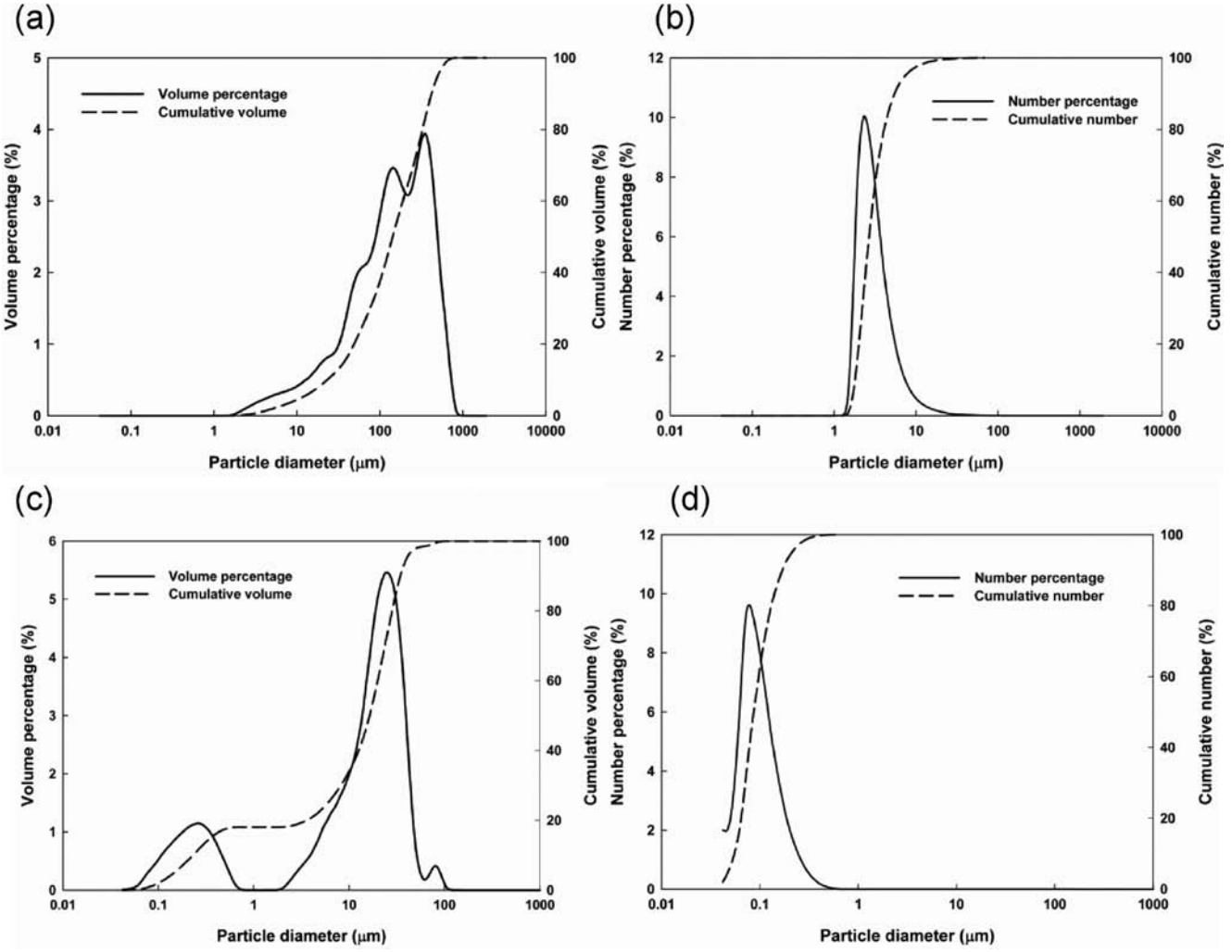

Figure 1. Particle size distributions of blended goji in (a) volume and (b) number and media-milled goji in (c) volume and (d) number.

Animal Care and Handling. Six-week-old male BALB/c mice were purchased from the animal center of the National Applied Research Laboratories in Taipei, Taiwan. After being received, mice were kept in a pathogen-free environment maintained at 21 ± 2 °C, 40−60% relative humidity, and 12 h intervals of light/dark cycle. Mice were fed tap water and Rodent Chow diet (Nutrition International, Brentwood, MO). Mice were grouped into three groups (n = 6 in each group) with minimal difference in total weight among the three groups. Media-milled and blended goji or phosphate-buffered saline (PBS) (as control group) were orally administered at 300 mg/kg body weight once daily for 7 days. Body weights of mice were measured before oral administration every day during experiments. Mice were euthanized by cervical vertebra dislocation, and spleens were excised and immediately stored in liquid nitrogen for further analysis.

Total RNA Isolation and Oligomicroarray Analysis. Total RNA was extracted from spleen using Trizol reagent (Invitrogen, USA) with the method of TrizoL RNA isolation protocol and then was purified using an RNeasy Mini Kit (Qiagen, Germany). The RNA samples were lysed in a denaturing buffer (RLT) containing guanidine−thiocyanate to immediately inactivate RNases and to ensure purification of intact RNA. Ethanol was added to provide binding conditions, and the sample was then transferred to an RNeasy Mini spin column coupled with a microcentrifuge tube. Total RNA bound to the membrane of the column. Centrifugation (8000*g*, 15 s) was employed to wash the spin column membrane. Contaminants were washed by adding an ethanol-containing buffer (RW1) and centrifugation (8000*g*, 2 min). The procedures were done in triplicate. The spin column was placed in a new microcentrifuge tube. Water was added, and then high-quality RNA was eluted in water (30−100 μL) by centrifugation (8000*g*, 1 min). The quantity of purified RNA was determined by measuring the absorption at 260 nm using an ND-1000 spectrophotometer (Nanodrop Technology, USA). The integrity of RNA was confirmed by Bioanalyzer 2100 (Agilent Technology, Santa Clara, CA). Total RNA (5 μg) was reversely transcribed using MMLV reverse transcriptase (Promega, Madison, WI) to prepare a cDNA. The resulting samples were diluted 40 times using DNase-free water. Six cDNAs in the same group were pooled together and stored at −20 °C until real-time PCR analysis.

Expression levels of various genes in the total RNA sample from mouse spleens were examined using an oligo-microarray system (Agilent Technologies). In brief, 0.5 μg of total RNA was amplified using a Fluorescent Linear Amplification Kit (Agilent Technologies) and labeled with either Cy3-CTP or Cy5-CTP (CyDye, PerkinElmer, Boston, MA) during the in vitro transcription process. Tested RNA was labeled with Cy5, and control RNA was labeled with Cy3. Cy-labeled cRNA (0.825 μg) was fragmented to an average size of 50−100 nucleotides by incubation with fragmentation buffer (G4122F-014868, Agilent Technologies) at 60 °C for 30 min. Correspondingly fragmented labeled cRNA was then pooled and hybridized to Agilent Whole Mouse

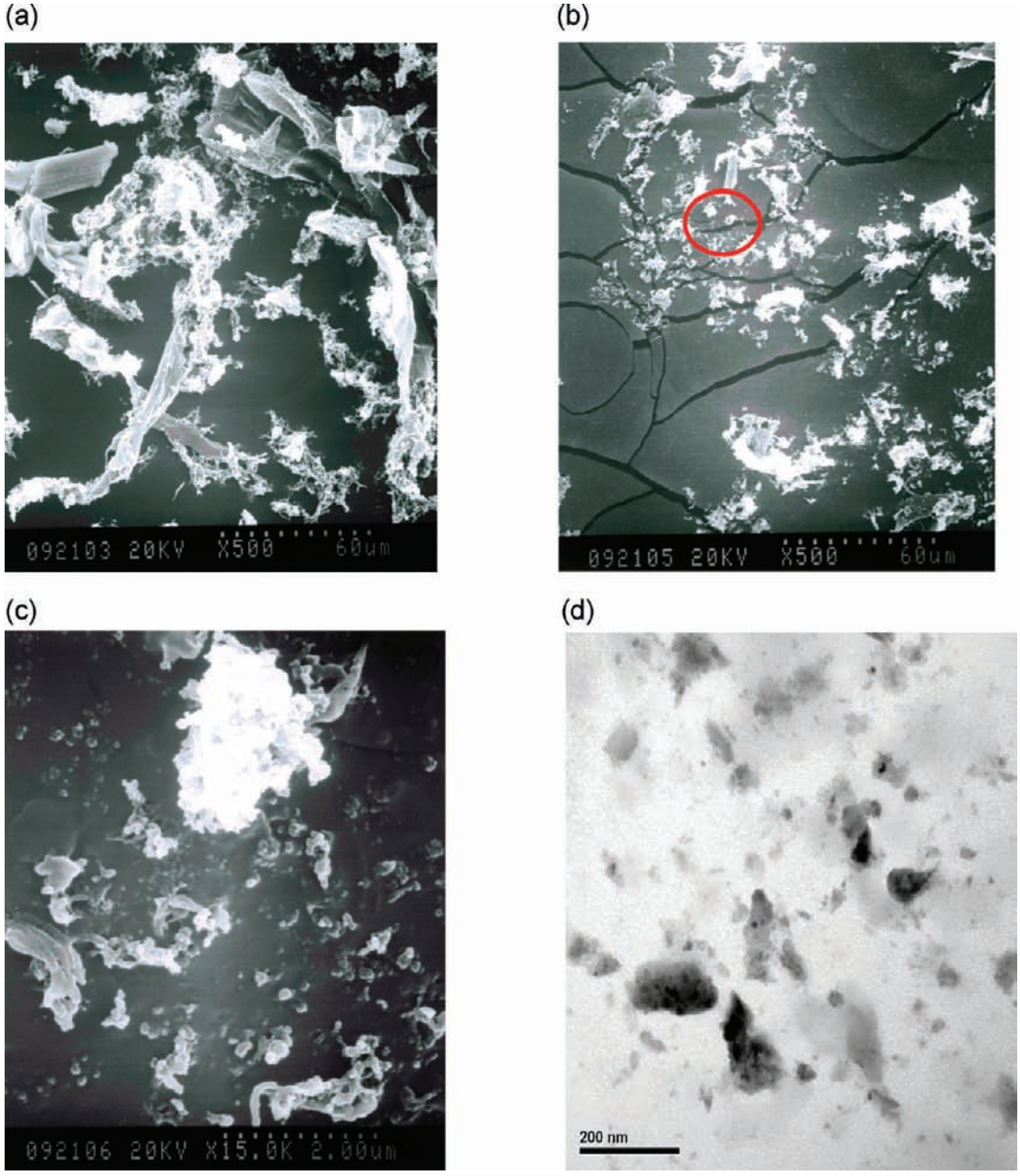

Figure 2. SEM of (a) blended and (b) media-milled goji suspension; (c) enlargement of the circled portion in panel b; and (d) TEM of media-milled goji suspension.

Genome 4x44k oligo-microarray (Agilent Technologies) at 65 °C for 17 h. After a final wash, the microarray slides were dipped into stabilization and drying solution (Agilent Technologies), which was developed for Agilent's oligo-based microarrays to prevent degradation of cyanine-5. The slides were removed from the solution and then dried by nitrogen gun blowing. Microarrays were scanned with an Agilent microarray scanner (Agilent Technologies) at 535 nm for Cy3 and at 625 nm for Cy5. Scanned images were analyzed by Feature Extraction Software 9.5.3 (Agilent Technologies). Image analysis and normalization software (Agilent Technologies) were used to quantify signal and background intensity for each feature, substantially to normalize the data by rank consistency filtering LOWESS method.[28]

Quantitative Real-Time PCR. Quantitative real-time PCR was conducted by SYBR Green I reaction. The above diluted cDNA (1.875 ng) was placed into each well to establish both standard curves of target mRNA. Gene-specific primers (0.75 μM/each primer) were added to each well. The forward and reverse primers used for TNF were 5′-TTGTCTTAATAACGCTGATTTGGT-3′ and 5′-GGGAGCAGAG-GTTCAGTGAT-3′; for Nfkb1, 5′-ATGGAATTAGCCCCAGGAAT-3′ and 5′-TGCTTTTACCCATCTTGCTG-3′; for caspase-3, 5′-TT-TCCACGCAAAGAAACAGA-3′ and 5′-CACCCCCAATCATTCCT-CTA-3′; for Bcl-2, 5′-CCCTGAAAAAGCCAAGAAAA-3′ and 5′-TCC-TGTGGGATGTCAACAAA-3′; and for APAF-1, 5′-CTTCTTTATG-GTGCTGAAGATTGA-3′ and 5′-GGTGGAGTGCCTGTCTAGTGT-3′. Internal controls were β-actin primers. cDNA was amplified using Roche LightCycler 1.5 (Indianapolis, IN) at the following conditions: 95 °C for 10 min and then 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 10 s, corresponding to the denaturation, primer annealing, and primer extension, respectively. During the amplification, a reaction product melt curve was established to provide evidence for a specific

Table 1. Proximate Composition[a] of Goji Suspensions

component	content (%)	
	blended	media-milled
crude ash	5.54 ± 0.18	4.49 ± 0.04
crude lipid	2.72 ± 0.50	2.21 ± 0.38
crude protein	14.72 ± 0.37	13.51 ± 0.60
dietary fiber	13.25 ± 1.03 a	16.53 ± 1.35 b
insoluble	9.71 ± 1.09	9.91 ± 1.41
soluble	3.54 ± 1.04 a	6.63 ± 0.29 b

[a] All results are presented on dry weight basis as the mean ± standard deviation (n = 3). Different lower case letters (in a and b) in the same row indicate significantly different at $p < 0.05$ analyzed by Duncan's multiple-range test.

Table 2. Bioactive Compounds[a] of Blended and Media-Milled Goji

sample	mg/g dry weight	
	blended	media-milled
total carotenoids	4.27 ± 0.03 a	5.81 ± 0.05 b
β-carotene	0.04 ± 0.01 a	0.19 ± 0.07 b
zeaxanthin	2.97 ± 0.02	3.95 ± 0.50
crude polysaccharides	15.69 ± 1.42 a	21.37 ± 1.52 b

[a] Mean ± standard deviation (n = 3). Different lower case letters (in a and b) in the same row indicate significantly different at $p < 0.05$ analyzed by Duncan's multiple-range test.

PCR product.[29] For each gene, the relative levels of transcripts were normalized to the level of β-actin (as an internal control). Relative expression levels were calculated using the equations[30]

$$\text{fold change} = 2^{-\Delta\Delta Ct} \quad \text{when} \quad \Delta\Delta Ct \leq 0 \tag{2}$$

$$\text{fold change} = -2^{\Delta\Delta Ct} \quad \text{when} \quad \Delta\Delta Ct > 0 \tag{3}$$

where $\Delta Ct = Ct_{(\text{target gene})} - Ct_{(\text{internal control gene})}$, $\Delta\Delta Ct_{(\text{media-milled})} = \Delta Ct_{(\text{media-milled})} - \Delta Ct_{(\text{control})}$, and $\Delta\Delta Ct_{(\text{blended})} = \Delta Ct_{(\text{blended})} - \Delta Ct_{(\text{control})}$. The result of quantitative RT-PCR was presented as the mean ± SD, with its significance evaluated by one-way ANOVA at $P < 0.05$.

RESULTS AND DISCUSSION

Particle Size Distribution and Microscopic Observation. The blended goji yielded a volume-average diameter of 144 ± 101 μm (Figure 1a) with a number-average diameter of 3.58 ± 3.80 μm (Figure 1b). The wide size distribution from 1 to 1000 μm indicated that there existed large particles. In addition, intact seeds were observed at the bottom of the suspension, indicating that blending did not break seeds, which were elliptical in shape with a semimajor axis of about 0.15 cm and a semiminor axis of about 0.1 cm. However, the size distribution did not indicate the presence of those large particles due to fast precipitation of seeds during measurement. The volume-average diameter of media-milled goji suspension was found to be 18.3 ± 14.8 μm (Figure 1c) with a number-average diameter of 100 ± 70 nm (Figure 1d). More than 60% of particles ranged from 1 to 100 nm, and most particles were smaller than 1 μm in number-size distribution. Nevertheless, the presence of a few large particles resulted in greater volume-average diameter. By comparison of panels b and d of Figure 1, it can be seen that 1 μm appeared to be the borderline for the size of blended and media-milled samples. Most particles from blended sample were >1 μm, and milling resulted in particles smaller than 1 μm. The data showed that nano/submicrometer goji particles were obtained by using media-milling. The size of particle was further confirmed by SEM and TEM. Blended sample yielded particles in the micrometer scale with irregular shape and wide distribution of size (Figure 2a). Media-milling eliminated large particles (Figure 2b). The size of particle was not affected by the cracking of tape during observation. Enlargement of a portion of Figure 2b revealed that there existed many particles smaller than 100 nm with shape approaching spherical (Figure 2c). TEM confirmed that particles were smaller than 200 nm and that some particles were smaller than 100 nm (Figure 2d).

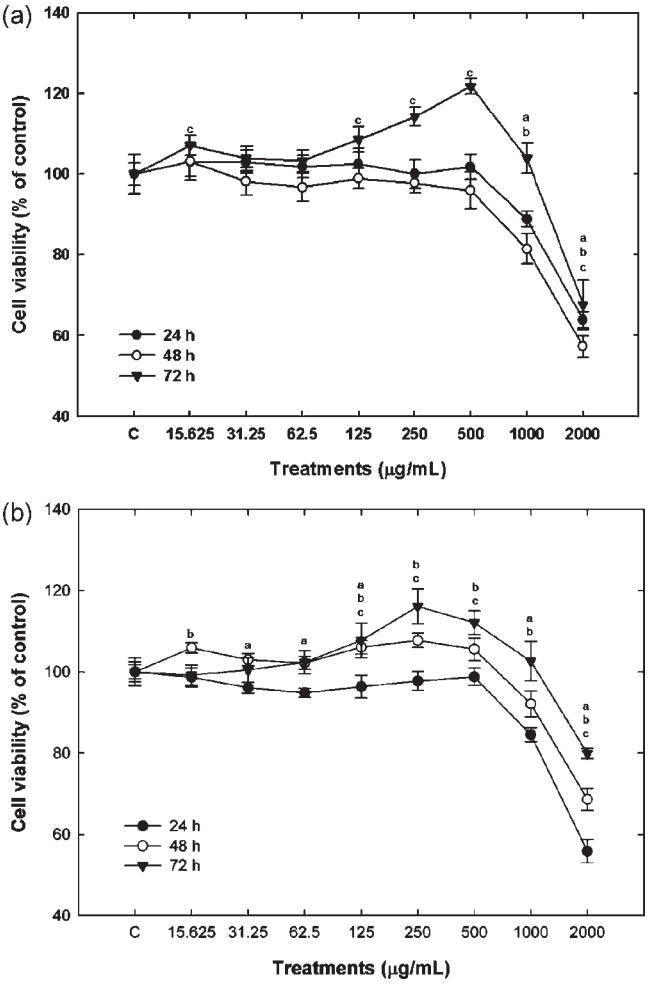

Figure 3. Cell viability of rat normal small intestinal cell line (IEC-6) treated with (a) blended goji and (b) media-milled goji for 24−72 h. Each value represents the mean ± standard deviation. Symbols indicate significant difference between control and treatments at $P < 0.05$, in which a, b, and c were for 24, 48, and 72 h, respectively.

Contents of Bioactive Compounds. The blended and media-milled goji suspensions appeared to have similar compositions, except for soluble fiber (Table 1). The significant increase in soluble fiber in media-milled goji was due to the impacting and

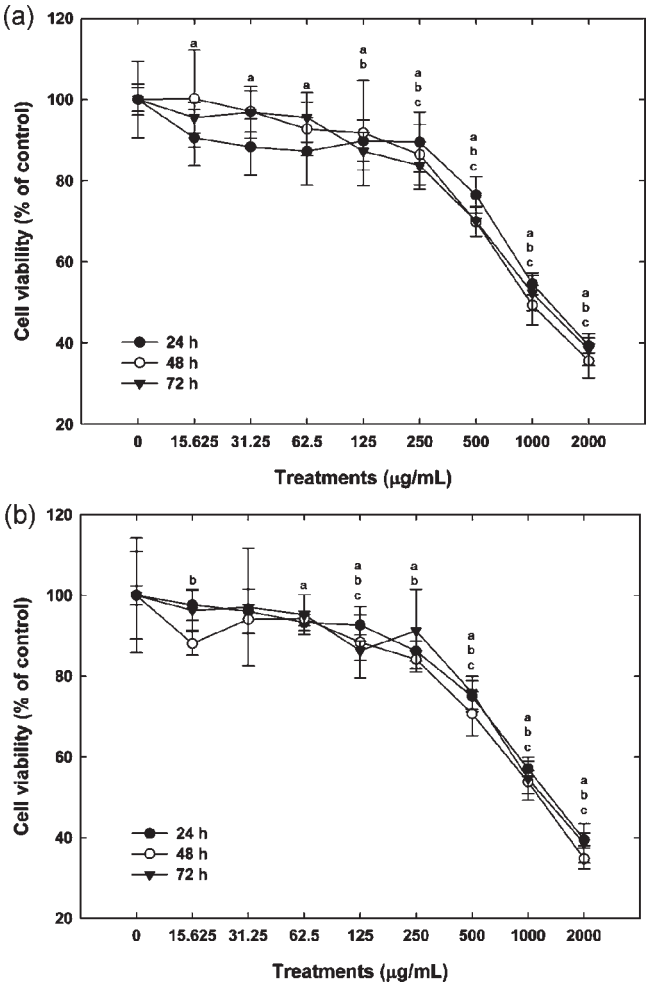

Figure 4. Cell viability of human colon adenocarcinoma cell line (Caco-2) treated with (a) blended goji and (b) media-milled goji for 24−72 h. Each value represents the mean ± standard deviation. Symbols indicate significant difference between control and treatments at $P < 0.05$, in which a, b, and c were for 24, 48, and 72 h, respectively.

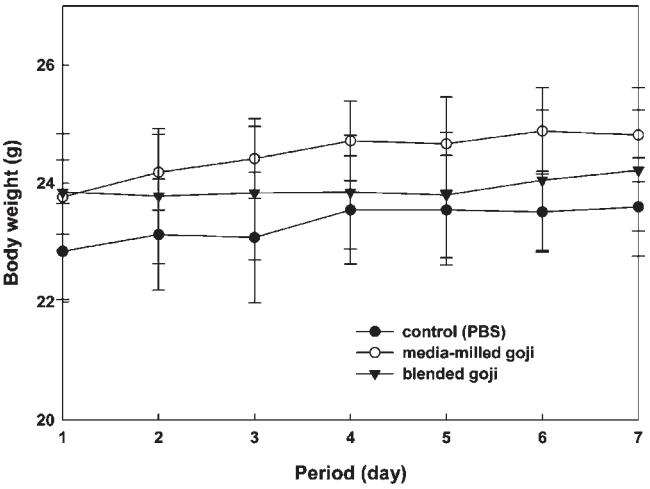

Figure 5. Average body weights of BALB/c mice ($n = 6$) during experiments. Each value represents the mean ± standard deviation. PBS stands for phosphate-buffered saline.

shearing force during media-milling, which also resulted in more bioactive compounds, including total carotenoids, β-carotene, and crude polysaccharides (Table 2). Among these three compounds, the increase in β-carotene was the greatest (>4 times). Media-milling resulted in a >35% increase in both total carotenoids and crude polysaccharides. The data indicated that the contents of total carotenoids, β-carotene and zeaxanthin, were in reported ranges.[3] The content of polysaccharide is reported to range from 1.7 to 40%.[1,31−34] Media-milling also resulted in a significant increase in polysaccharide. It appeared that media-milling was an alternative to solvent extraction to increase the contents of bioactive compounds and dietary fibers. Although the increase in zeaxanthin was not statistically significant, the data indicated that media-milled goji might enhance the efficacy of goji products.

Cell Viability Assay. Neither blended nor media-milled goji affected the viability of IEC-6 cells until the addition level was >500 μg/mL (Figure 3). Although there existed a significant increase in viability when blended goji was increased from 62.5 to 500 μg/mL for 72 h of incubation, neither blended nor media-milled goji exhibited any toxicity to IEC-6 cells. When the addition level was >500 μg/mL, both blended and media-milled goji resulted in a dramatic drop in cell viability. For Caco-2 cells, adding blended goji at 15.6 μg/mL resulted in a decrease in cell viability to 90.6% (Figure 4a). Cell viability decreased gradually with continued addition of blended goji to 250 μg/mL. When the addition level was >250 μg/mL, cell viability decreased dramatically. Addition of media-milled goji from 15.6 to 250 μg/mL also resulted in a gradual decrease in viability of IEC-6 cells (Figure 4b). Again, cell viability dropped dramatically when the addition level was >250 μg/mL. Blended and media-milled goji exhibited similar effects on the viability of cells. The presence of nanoparticles in media-milled goji did not exhibit any unusual effect on cell viability. The LC_{50} values of both samples for IEC-6 (normal small intestinal epithelial cell from rat) were >2 mg/mL, whereas the LC_{50} of both samples for Caco-2 were about 1 mg/mL. The LC_{50} of bog bilberry extracts for Caco-2 and 3T3-L1 (nonmalignant murine fibroblast cell line) were 0.390 ± 0.30 and 0.214 ± 0.02 mg/mL, respectively.[35] Both blended and media-milled goji showed lower cytotoxicity than bog bilberry. Nevertheless, both blended and media-milled goji affected differently cell viability of malignant and nonmalignant cell lines.

Body Weight. Figure 5 shows average the body weight of mice during experiments. Initially, the difference in average body weight among the three groups was <1 g (<5% of body weight). The grouping did not result in significant difference in average body weight. The average body weight of both the control and media-milled goji group increased slightly, 0.75 and 1.05 g, respectively. The blended-goji group did not gain weight until day 6. Our data show that the administration of blended and media-milled goji did not result in an adverse effect on the growth of mice.

Gene Expression from Spleen. From the results of oligo-microarray analysis, there were 785 and 771 genes up-regulated and 176 and 166 genes down-regulated by blended and media-milled goji, respectively. There were 13 genes affected related to cell survival (Table 3). After comparing different pathways, we were interested in apoptosis and survival TNFR1 signaling pathway.[36] Five genes (Bcl-2, Nfkb1, TNF, Casp3, and Apaf1) discussed in this study are involved in this pathway. For those genes being up-regulated, media-milled goji yielded greater fold changes than blended goji. The expression fold changes of Bcl-2

Table 3. Fold Change[a] in Expression Intensities of Genes Related to Cell Survival

fold change[b]				
M	B	gene symbol	gene product	accession no.
2.30	2.12	*Bcl2*	B-cell leukemia/lymphoma 2	AK077913
2.04	1.72	*Nfkb1*	nucleus factor of κ light chain enhancer gene in B cell	BC050841
1.98	1.53	*Cd28*	mouse musculus CD28 antigen	NM_007642
1.74	1.56	*Tnf*	mouse musculus tumor necrosis factor	NM_013693
1.63	1.97	*Bcl10*	B-cell leukemia/lymphoma 10	AK080820
1.59	1.81	*Mapk8*	mitogen activated protein kinase 8	AK163829
1.55	1.51	*Gadd45b*	*Mus musculus* growth arrest and DNA-damage-inducible 45 β	NM_008655
1.50	1.79	*Cd69*	*Mus musculus* CD69 antigen	NM_001033122
−1.56	−1.61	*E2f1*	*Mus musculus* E2F transcription factor 1	NM_007891
−1.59	−1.69	*Mmp8*	*Mus musculus* matrix metallopeptidase 8	NM_008611
−1.59	−1.64	*Chek2*	*Mus musculus* CHK2 checkpoint homologue	NM_016681
−1.64	−1.72	*Casp3*	caspase 3, apoptosis-related cysteine peptidase	U49929
−1.79	−1.49	*Apaf1*	apoptotic peptidase activating factor	NM_001042558

[a] Each value represents the mean of six measurements ($n = 6$). [b] Fold change: expression level of target gene (tested mice)/expression level of target gene (control mice). M, mice fed media-milled goji; B, mice fed blended goji.

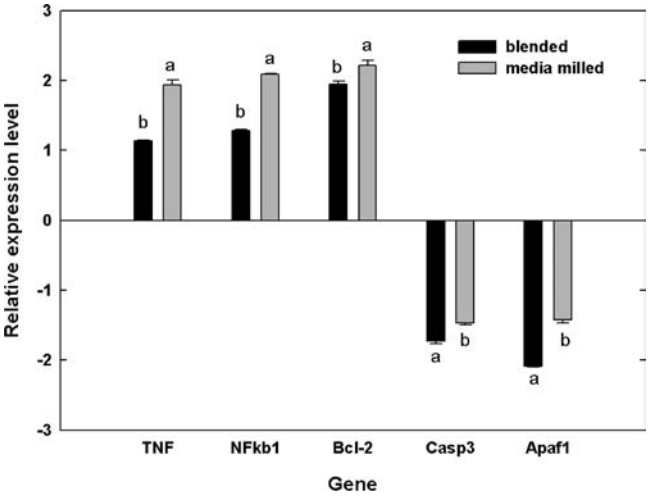

Figure 6. Relative expression of different genes in spleen of mice fed blended or media-milled goji suspensions. Each value presents the mean ± standard deviation. Different lower case letters (a, b) for the same gene indicate significant difference at $P < 0.05$ as analyzed by Duncan's multiple-range test.

and Nfkb1 in spleens of mice fed media-milled goji were 2.30 and 2.04 times, respectively, those of the control group (mice fed phosphate-buffered saline). Compared with blended goji, size reduction resulted in about 9 and 19% increases in fold change of Bcl-2 and Nfkb1, respectively. As Apaf1 was down-regulated, size reduction reduced >20% fold change. Our data illustrate that size reduction via media-milling significantly affected the fold changes of gene expressions in mouse spleen.

Results of quantitative real-time PCR were used to corroborate the data of oligomicroarray analysis. As mice were fed with goji, the mRNA expression levels of TNF, Nfkb1, and Bcl-2 were significantly up-regulated, whereas the mRNA expression levels of Casp3 and Apaf-1 were both down-regulated (Figure 6). Comparison of blended and media-milled goji showed that up-regulation on TNF, Nfkb1, and Bcl-2 by media-milled goji was significantly greater than that by blended goji. The relative expression levels of both TNF and Nfkb1 were increased >60% due to size reduction. Media-milling resulted in only a 13.8% increase in the relative expression level of Bcl-2. Nevertheless, the down-regulation on Casp3 and Apaf-1 was reduced by size reduction. The relative expression level of APAF-1 was reduced 46.9% by media-milling. TNF was reported to induce either apoptosis or cell survival, depending on the balance between anti- and pro-apoptotic pathways.[37] The death signals seem to be associated with the activation of both caspase and JUN kinase pathways, whereas the survival signals are mediated via the activation of the NF-κB pathway.[38] The DNA binding activity of NF-κB is rapidly induced in all cell types in response to pro-inflammatory cytokines and the byproducts of microbial and viral infection.[39] The activation of NF-κB has been shown to regulate both innate and adaptive immune responses and to be associated with cell survival.[40] NF-κB has been shown to inhibit apoptosis.[40] A primary response to inflammation is an increased survival signal, and the principal mechanism for the extended survival is through induction of anti-apoptotic Bcl-2 family proteins.[41] NF-κB has been shown to activate the transcription of inflammation-related genes, such as TNF-α and Bcl-2.[42] Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins during apoptosis. Caspases exist as inactive proenzymes that result in a sequential activation of caspases and play a central role in cell apoptosis.[43] Apoptotic protease activating factor 1 (Apaf-1) is an apoptotic regulatory protein that can bind to cytochrome *c*. Apaf-1 triggers the activation of that caspase signaling pathway and plays a key role in the intrinsic pathway of apoptosis.[44] Goji appeared to exhibit an anti-apoptosis effect, which was enhanced by media-milling.

Polysaccharides of *L. barbarum* (LBP) were found to increase the anti-apoptotic protein Bcl-2 level in epithelial cells of the whole lens, probably due to direct antioxidative effects of LBP.[45] One fraction of LBP was found to stimulate proliferation of isolated splenocytes and B-lymphocytes by up-regulating NF-κB and activator protein-1 (AP-1) expression.[46] LBP shows the neuroprotective activities via significant reduction or attenuation of the activity of caspase-3 triggered by Aβ peptide.[8,47] Ho et al.[48]

showed that LBP significantly attenuates homocysteine-induced neuronal cell death and apoptosis in primary cortical neurons via suppression of caspase-3 activity.

The contents of bioactive compounds, total carotenoids, β-carotene, zeaxanthin, and crude polysaccharides were greater in media-milled goji than in blended goji. It was reasonable to assume that media-milled goji exhibited a greater effect on gene expression than blended goji. On the basis of the above discussion, we suggest that TNF-α-mediated response was induced by goji, and then survival signals were mediated via the activation of the NF-κB pathway, including up-regulation of Bcl-2 and down-regulation of effector caspase (for example, caspase-3) and Apaf-1. Our results provide the evidence that TNF-α-mediated response was regulated by goji and that survival signals were then mediated via the activation of the NF-κB pathway, including further up-regulation of Bcl-2 and down-regulation of caspase-3 and Apaf-1. These regulatory effects were related to modulating apoptosis signaling in mouse spleen. These phenomena might be related with cytoprotective activity and can be further applied to the development of nutraceutical products from food materials. However, further studies are needed to confirm the concept.

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ABBREVIATIONS USED

AMD, age-related macular degeneration; ANOVA, analysis of variance; AOAC, Association of Official Analytical Chemists; Caco-2, human colorectal adenocarcinoma; cDNA, complementary DNA; cRNA, complementary RNA; CTP, cytosine triphosphate; Cy3, cyanine3; Cy5, cyanine5; FBS, fetal bovine serum; IEC-6, rat intestine epithelial cell; JUN, jun proto-oncogene; LBP, polysaccharides of *Lycium barbarum*; LC_{50}, lethal concentration 50; LOWESS, locally weighted scatterplot smoothing; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, real-time polymerase chain reaction; SAS, Statistical Analysis System; SEM, scanning electron microscopy; TEM, transmission electron microscopy; UV, ultraviolet; YTZ, yttria-stabilized tetragonal zirconia.

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