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Anti-inflammatory Effects of Isoflavone Powder Produced from Soybean Cake

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Soybean cake, a byproduct obtained during the processing of soybean oil, has been shown to be a rich source of isoflavones. The objectives of this study were to use soybean cake as raw material for processing into powder and to evaluate the anti-inflammatory activity. Eleven treatments, including powders of malonylglucoside, glucoside, acetylglucoside, aglycone, ISO-1, and ISO-2, as well as genistein standard, γ-PGA, control, normal, and PDTC, were used for evaluation. A total of 77 mice were each provided daily with tube feeding for 4 weeks at a dose of 0.3 mL of aqueous solution from each treatment, and inflammation was induced with intraperitoneal injection of 1 mg/kg of body weight lipopolysaccharide (LPS). Results showed that all of the isoflavone powders and genistein standard were effective in inhibiting LPS-induced inflammation, lowering leukocyte number in mice blood and reducing production of IL-1β, IL-6, NO, and PGE_2 in both peritoneal exudate cell supernatant and peritoneal exudate fluid. All of the isoflavone treatments failed to retard T cell proliferation; however, both ISO-1 and ISO-2 could inhibit B cell proliferation. The difference in anti-inflammatory activity was minor between any of the isoflavone treatments.

KEYWORDS: Soybean cake; isoflavone; anti-inflammation; animal model; functional components

INTRODUCTION

Isoflavone, a major class of flavonoids mainly present in soybean, has been shown to possess antioxidant activity, reduce risk of cardiovascular disease, and inhibit cancer cell growth (*1–4*). Several recent studies also demonstrated that isoflavone may exhibit anti-inflammatory activity (*5–7*). Inflammation, a kind of innate immunity, can result in killing or degradation of outer microorganisms by neutrophils in blood and macrophage in tissues through combination with polysaccharides on the microorganism's surface (*8*). Pus can thus be formed after the death of neutrophils that have entered damaged tissues because of infection. Meanwhile, macrophages can secrete several soluble proteins called cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), and tumor necrosis factor (TNF-α). Among these cytokines, IL-1, IL-6, and TNF-α have attracted more attention as they can be localized to the infected tissue, manifested systemically throughout the body, and cause vasodilation as well as inflammation symptoms such as redness, swelling, heat, and pain (*8*). Other cytokines, such as IL-8 and IL-12, were seldom investigated. Both IL-1 and TNF-α have been reported to increase expression of intercellular adhesion molecular-1 (ICAM-1) and vascular cell adhesion molecular-1 (VCAM-1), resulting in adhesion of neutrophil, monocyte, and macrophage to vessel wall and subsequent inflammation of infected tissues (*8*). In addition to cytokine, macrophage can secrete inflammation mediators such as prostaglandin E_2 (PGE_2) and nitric oxide (NO), causing sepsis, sepsis shock, and systemic inflammatory response syndrome (*9*). Also, both chronic disease and autoimmune disease can thus be deteriorated (*10, 11*).

Only several studies dealing with the inhibition of inflammation by isoflavones are available in the literature. Yankep et al. (*12*) reported that the isoflavone derivative griffonianone D extracted from *Millettia griffoniana* may retard edema on the right hind paw induced by injection of carrageenan. Paradkar et al. (*13*) found that with an isoflavone-containing diet intraperitoneal lipopolysaccharide (LPS) injection in mice led to a decrease in the liver antioxidant glutathione level and prevention of the inflammation-associated induction of metallothionein in the intestine. In a later study Hall et al. (*2*) demonstrated that dietary isoflavones have beneficial effects on C-reactive protein (CRP) concentrations, but not on other inflammatory biomarkers of cardiovascular disease risk in postmenopausal women, and may improve VCAM-1 response in an ERβ gene polymorphic subgroup. Similarly, the CRP level could be raised in the blood of patients with end-stage renal disease, but both IL-6 and TNF-α were unaffected (*6*). Several papers also suggested that genistein could activate peroxisomal proliferator-activated receptor-γ (PPAR-γ) and in turn retard adhesion of monocyte to human vascular endothelial cells, a key step within the inflammatory cascade leading to athero-

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genesis, which may be associated with the A ring in the isoflavone structure (*5, 14*). In a recent study Park et al. (*7*) further pointed out that isoflavones extracted from *Pueraria thunbergiana* could suppress LPS-induced release of NO and TNF-α in primary cultured microglia and BV2 microglial cell lines.

Soybean cake, a byproduct obtained during the processing of soybean oil, has been shown to be a rich source of isoflavones and other functional components such as saponins and phenolic compounds (*3, 15*). It would be a great advantage to the health food industry if the isoflavones could be isolated from soybean cake and proved to be anti-inflammatory. As most studies dealing with the effect of isoflavones on anti-inflammation are focused on cell and chronic inflammation, there is a paucity of data in terms of inhibition of acute inflammation using animal models. The objectives of this research were thus to study the anti-inflammatory effect using LPS-induced mice in the presence of various isoflavone powders produced from soybean cake.

MATERIALS AND METHODS

Materials. Reagents including sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium bicarbonate, potassium bicarbonate, ammonium chloride, EDTA-2Na, sodium nitrite, lipopolysaccharide (from *Escherichia coli* O55:B5 and purified by phenol extraction), lipopolysaccharide (from *E. coli* O26: B6 and purified by gel filtration chromatography), concanavalin A, ammonium pyrrolidinedithiocarbamate (PDTC), sulfanilamide, *N*-1-naphthylethylenediamide dihydrochloride, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Culture medium RPMI-1640, L-glutamin, *N*-2-hydroxyethylpiperazine ethanesulfonic acids (HEPES), fetal bovine serum (FBS), β-mercaptoethanol, penicillin−streptomycin, trypan blue stain (0.4%), and Hank's balanced salt solution (HBSS) were from HyClone Co. Isoflavone standard genistein was from LC Laboratories (Woburn, MA). The DuoSet ELISA development system was from R&D Systems Co. (Minneapolis, MN) and BD Biosciences Co. (San Diego, CA). The PGE_2 test kit was from Assay Designs Co. (Ann Arbor, MI).

Production of Isoflavone Powder. Initially the isoflavone extract was prepared from soybean cake using a method described by Kao et al. (*15*) and was used as a basis for production of isoflavone powder. Briefly, a total of 50 g of soybean cake was mixed with 150 mL of water/ethanol (1:1, v/v) in a 250 mL flask and shaken for 2 h, after which the mixture was centrifuged at 6000 rpm for 20 min and the supernatant was collected. After filtration through a glass filter paper, a 75 mL filtrate was collected and poured into a glass column (375 × 45 mm i.d.) containing 200 g of adsorbent Diaion HP-20, which was previously activated with 1 L of ethanol and 1 L of distilled water. Impurities were first removed with 400 mL of deionized water, followed by elution of malonylglucoside with 900 mL of ethanol/water (15:85, v/v) and glucoside with 3300 mL of ethanol/water (27:73, v/v). Both acetylglucoside and aglycone were eluted with 200 mL of ethanol/water (70:30, v/v) and 400 mL of 100% ethanol, respectively. However, because of partial overlapping, acetylglucoside and aglycone fractions were combined and poured into a Yamazen Hi-Flash silica gel column (170 × 48 mm i.d.), and both were eluted separately with a solvent system of hexane/isopropanol/ethanol (8:9:1, v/v/v).

For production of malonylglucoside powder, the malonylglucoside fraction was collected 15 times and evaporated to dryness. Then the residue was dissolved in 200 mL of ethanol/water (1:1, v/v) and mixed with 80 mL of aqueous 0.01% poly(γ-glutamic acid) (γ-PGA) solution. After homogenization for 10 min, the solution was placed into a −30 °C freezer for 24 h and subjected to freeze-drying. A 6.9 g powder was produced and found to contain malonylglucoside at 43489 μg/g after HPLC analysis, and a portion (0.53 g powder) was collected and dissolved in 6.9 mL of deionized water with a final concentration of malonylglucoside at 3.33 mg/mL. Likewise, for production of glucoside powder, the glucoside fraction was collected eight times and evaporated to dryness. Again, the residue was dissolved in 200 mL of ethanol/water (1:1, v/v), mixed with 80 mL of aqueous 0.01% γ-PGA solution, and homogenized for 10 min. The mixture was then frozen in a −30 °C freezer for 24 h, and a powder product of 6.2 g was obtained after freeze-drying, in which the glucoside was present at a level of 47938 μg/g by HPLC analysis. A portion (0.48 g) of powder was collected and dissolved in 6.9 mL of deionized water for a final content of glucoside at 3.33 mg/mL.

Prior to production of acetylglucoside and aglycone powder, soybean cake was heated in an oven for conversion of malonylglucoside to its corresponding acetylglucoside and aglycone (*16*). In practice, a 100 g sample of soybean cake was spread onto a plate uniformly, which was preheated to 200 °C in an oven. After heating for 5 min, the soybean cake was turned over, and heating was continued for another 5 min; then the cake was cooled in a −20 °C freezer. This procedure was repeated 10 times, and a total of about 950 g of soybean cake was obtained. For powder production, a 50 g soybean cake sample was mixed with 150 mL of water/ethanol (1:1, v/v) and shaken for 2 h, after which this mixture was centrifuged for 20 min and filtered to obtain about 75 mL of supernatant. This procedure was repeated 30 times, and a total of about 2250 mL of supernatant was collected. A portion of the extract (50 mL) was poured into a Yamazen Hi-Flash (170 × 48 mm i.d.) silica gel column, and both acetylglucoside and aglycone were eluted separately with a solvent system of hexane/isopropanol/ethanol (8:9:1, v/v/v) with a flow rate at 20 mL/min. The acetylglucoside fraction was collected 40 times and the aglycone fraction 30 times, both of which were evaporated to dryness; the residues were dissolved in 200 mL of ethanol/water (1:1, v/v), followed by mixing with 80 mL of 0.01% aqueous γ-PGA solution and homogenizing for 10 min. Next, the mixtures were frozen at −30 °C for 24 h and subjected to freeze-drying, after which powder products of about 6.1 g of acetylglucoside and 6.3 g of aglycone were generated. After HPLC analysis, the former was found to contain acetylglucoside at 46398 μg/g, and the latter contained aglycone at 42101 μg/g. A portion of acetylglucoside powder (0.50 g) and aglycone powder (0.53 g) was dissolved in 6.9 mL of deionized water separately to obtain final concentrations of 3.33 mg/mL for both.

For production of ISO-1 powder, a 200 mL soybean cake extract (without column chromatography) was mixed with 80 mL of 0.01% aqueous γ-PGA solution and homogenized for 10 min, after which the mixture was frozen at −30 °C for 24 h and then freeze-dried. A powder product of 9.5 g was produced with a level of total isoflavone at 43839 μg/g by HPLC analysis. A portion (0.52 g) of powder was dissolved in 6.9 mL of deionized water to obtain the total isoflavone content at 3.33 mg/mL.

For production of ISO-2 powder, a 1.2 g powder samples of malonylglucoside, glucoside, acetylglucoside, and aglycone were collected separately and mixed for a total amount of 4.8 g. After HPLC analysis, a level of total isoflavone (45232 μg/g) was found and a portion (0.51 g) of powder was dissolved in 6.9 mL of deionized water for a final concentration of total isoflavone of 3.33 mg/mL.

For preparation of genistein solution, a 23 mg genistein standard was dissolved in 100 mL of dimethyl sulfoxide (DMSO) and mixed with 6.9 mL of deionized water for a final concentration of genistein of 3.33 mg/mL.

For preparation of aqueous γ-PGA solution, a 23 mg γ-PGA (sodium type, molecular mass = 880 kDa) was mixed with 6.9 mL of deionized water for a concentration of 3.33 mg/mL.

Functional Components in Isoflavone Powder. The contents of phenolic acids, total flavonoids, total phenolic compounds, α-tocopherol, ascorbic acid, and soyasaponins in isoflavone powder were determined on the basis of a method described in a previous study (*3*).

Preparation of Reagents. A phosphate-buffered saline (PBS) solution (10 × PBS) was prepared by mixing 80.8 g of NaCl, 29.2 g of $Na_2HPO_4 \cdot 12H_2O$, 2.0 g of KH_2PO_4, and 2.0 g of KCl and dissolving in 1 L of deionized water, after which the mixture was diluted to an optimum concentration for use.

A CM-10 (complete medium containing 10% FBS) culture medium was prepared by mixing 1000 mL of membrane-filtered RPMI-1640 medium supplemented with 100 mL of decomplement FBS, 10 mL of penicillin (10000 units/mL)−streptomycin (10000 μg/mL), 10 mL of 200 mM L-glutamin, 10 mL of 1000 mM *N*-2-hydroxyethylpiperazine

ethanesulfonic acids (HEPES), and 1 mL of 5.78×10^{-2} M β-mercaptoethanol, after which a portion (3 mL) of the mixture was collected and cultured for 3 days to see if any pollution occurred.

A Hank's balanced salts solution (HBSS) was prepared by mixing 9.5 g of HBSS powder and dissolving in 1 L of deionized water, after which the mixture was adjusted to pH 7.2–7.3 with sodium bicarbonate and filtered through a 0.2 μm membrane filter.

An ammonium chloride−potassium lysis buffer (ACK lysis buffer) was prepared by mixing 0.2 g of $KHCO_3$, 1.65 g of NH_4Cl, and 0.2 mL of 0.1 M EDTA-2Na, after which the mixture was dissolved in 100 mL of deionized water, adjusted to pH 7.0, diluted to 200 mL, and filtered through a 0.2 μm membrane filter.

A concentration of 0.2 mg/mL lipopolysaccharide in 1× PBS was prepared, and 0.1 mL [1 mg/kg of body weight (BW)] was used to induce systemic inflammation with intraperitoneal injection.

A concentration of 20 mg/mL ammonium pyrrolidinedithiocarbamate (PDTC) in 1× PBS was prepared, and 0.1 mL (100 mg/kg BW) was used with intraperitoneal injection for control treatment.

A concentration of 5 mg/mL concanavalin A (Con A) in CM-10 culture medium was prepared to stimulate splenocyte proliferation and diluted to 5 μg/mL prior to use.

A concentration of 1 mg/mL LPS in CM-10 culture medium was prepared to stimulate proliferation of both splenocyte and peritoneal exudate cell and diluted to 10 and 20 μg/mL prior to use.

A mixture of 100 mg/mL LPS and 50 units/mL IFN-γ in CM-10 culture medium was prepared to stimulate nitric oxide formation in peritoneal exudate cell.

Experimental Animals. Each mouse was provided daily with tube feeding at a dose of 0.3 mL of aqueous solution from each treatment shown above, which is equivalent to a total isoflavone at 50 mg/kg of BW. The aqueous solution was prepared fresh every 3 days as a volume of 6.9 mL can be used for tube feeding of seven mice for 3 days. The female 8-week-old BALB/c mice (specific pathogen free) were procured from Taiwan BioLASCO Co. (Taipei, Taiwan). They were fed a chow diet (laboratory rodent diet 5001) obtained from LabDiet Co. (St. Louis, MO). All of the mice were housed in individual ventilation cages (IVC) with a temperature at 22 ± 2 °C and a relative humidity at 50–60% in the animal center of the Graduate Institute of Nutrition and Food Science, Fu Jen University. The body weight of each mouse was measured every week during the feeding period. After the mice acclimatized for 1 week, a total of 77 mice were divided into 11 groups with 7 each and administered the following aqueous solutions of powder by tube feeding at a dose of 50 mg/kg of BW for 4 weeks: (1) M group, 0.3 mL of aqueous solution of malonylglucoside powder for each mouse; (2) G group, 0.3 mL of aqueous solution of glucoside powder for each mouse; (3) Ac group, 0.3 mL of aqueous solution of acetylglucoside powder for each mouse; (4) Ag group, 0.3 mL of aqueous solution of aglycone powder for each mouse; (5) ISO-1 group, 0.3 mL of aqueous solution of isoflavone powder for each mouse; (6) ISO-2 group, 0.3 mL of aqueous solution of isoflavone powder from a combination of four isoflavone fractions for each mouse; (7) γ-PGA group, 0.3 mL of aqueous solution of γ-PGA for each mouse; (8) genistein group, 0.3 mL of aqueous genistein solution for each mouse; (9) control group, 0.3 mL of sterilized water for each mouse; (10) normal group, 0.3 mL of sterilized water for each mouse; (11) PDTC group, 0.3 mL of sterilized water for each mouse. After feeding for 4 weeks, the PDTC group was first given an intraperitoneal injection of PDTC at a level of 0.1 mL (100 mg/kg of BW), followed by injection of 0.1 mL of LPS (1 mg/kg of BW) to induce systemic inflammation for all groups 1 h later, with the exception of the normal group. All of the mice were sacrificed 4 h after injection of LPS.

Animal Sacrifice and Sample Collection. *Blood Collection.* After injection of PDTC and LPS, all of the mice were weighed and anesthetized with ether, and blood was collected from the retro-orbital plexus using a sterilized Pasteuer glass tube and transferred to a microcentrifuge tube containing heparin, followed by storage at 4 °C; leukocytes were counted within 12 h.

Peritoneal Exudate Fluid Collection. After blood collection, all of the mice were sacrificed with CO_2 inhalation and spread with 70% alcohol. The skin was lifted upward from the abdominal wall with tissue forceps, and an incision was snipped with scissors. Fur was stripped back, and the abdominal wall was lifted up with a forceps. A 1 mL HBSS solution was injected into the abdominal cavity gradually, and at the same time the abdominal cavity of the mouse was tapped gently to assist in mixing peritoneal exudate fluid and buffered solution. Approximately 0.7 mL of peritoneal exudate fluid was collected with a syringe, followed by centrifuging at 1500 rpm (4 °C) for 5 min, and the supernatant was collected and stored at −20 °C for use.

Peritoneal Exudate Cell Collection. After collection of peritoneal exudate fluid (0.7 mL), a 6 mL HBSS solution was injected into the abdominal cavity and the buffered solution containing peritoneal exudate cells was collected using a syringe. This procedure was repeated twice to obtain a slightly yellow buffered solution, followed by centrifuging at 1500 rpm (4 °C) for 5 min, and the supernatant was removed. A 1 mL CM-10 medium was added and combined with the peritoneal exudate cells from centrifuged peritoneal exudate fluid, after which cells were counted.

Splenocyte Collection. After collection of peritoneal exudate cells, mice were sprayed with 70% alcohol for sterilization. The peritoneal membrane was cut with a scissors, and the spleen was collected and weighed. Then the spleen was crushed and pestled gently so that cells were suspended into HBSS solution. After the connective tissue was precipitated, the suspension was pipetted into a tube for centrifuging at 1500 rpm (4 °C) for 5 min. The supernatant was removed, and 1 mL of ACK lysis buffer was added to lyse the erythrocytes and reacted for 1 min, followed by the addition of 5 mL of HBSS solution and centrifugation under the same condition. Likewise, the supernatant was removed, and 5 mL of CM-10 was added to the dispersed cell pellet for cell counting.

Peritoneal Exudate Cell Supernatant. Cells were seeded at 2×10^6 cell/well into a 24 well plate, followed by stimulation of cytokine with 10 μg/mL of LPS or of nitric oxide formation with a mixture of LPS (50 ng/mL) and IFN-γ (25 units/mL). After culture at 37 °C for 24 h, the solution was centrifuged at 2400 rpm (4 °C) for 10 min, and the supernatant was collected and stored at −20 °C for use.

Measurement of Cytokine and Inflammatory Mediator Levels by ELISA. Cytokine (IL-1β, IL-6, and TNF-α) levels in peritoneal exudate cell supernatant and peritoneal exudate fluid were determined by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D systems, BD Biosciences). Capture antibody was diluted with coating buffer (1× PBS) to concentrations of 4 μg/mL for IL-1β, 2 μg/mL for IL-6, and 1:250 (with PBS) for TNF-α, followed by the addition of 100 μL of each capture antibody to a 96-well plate and incubation at 4 °C overnight. Then each plate was washed with washing buffer (0.05% Tween 20 in 1× PBS, pH 7.2–7.4) three times, after which 200 μL of blocking buffer (5% sucrose in 1× PBS for IL-1β, 1% BSA in 1× PBS for IL-6, and 2% FBS in 1× PBS for TNF-α, pH 7.2–7.4) was added and incubated at room temperature for 2 h to block nonspecific binding. Next, each plate was washed four times with washing buffer (≥300 μL/well) to remove residual blocking buffer, and 100 μL of standard (1000, 500, 250, 125, 62.5, 31.3, and 15.6 pg/mL in blocking buffer) and samples were added to each well, followed by incubation at room temperature for 2 h and five washings with washing buffer (≥300 μL/well) to remove unreacted standards and samples. A 100 μL detection antibody [100 ng/mL for IL-1β, 200 ng/mL for IL-6 and 1:500 (with blocking buffer) for TNF-α] was added to each plate and incubated at room temperature for 2 h, followed by six washings with washing buffer (≥300 μL/well) to remove unreacted detection antibody. To each well was added 100 μL of peroxidase streptavidin−HRP (diluted with blocking buffer at 1:200), and then each plate was incubated for 20 min at room temperature, after which all plates were washed with washing buffer (≥300 μL/well) seven times to remove unreacted enzyme. Again, each well was added with 100 μL of tetramethylbenzidine (TMB) substrate solution, and each plate was incubated for 20 min at room temperature, after which a 50 μL stop solution (2.5% sulfuric acid) was added to terminate the reaction; the absorbance was measured at 450 nm with an ELISA reader. The concentrations of IL-1β, IL-6, and TNF-α were determined on the basis of the linear regression equations obtained from the standard curves.

Determination of PGE_2. The level of PGE_2 was measured on the basis of a competitive immunoassay, and a standard procedure was

followed according to the Assay Designs Co. Prior to analysis, the peritoneal exudate fluid and peritoneal exudate cell supernatant without LPS stimulation were diluted to 1:10 with assay buffer, whereas the peritoneal exudate cell supernatant stimulated with LPS was diluted to 1:20. One hundred microliters of standard (2500, 1250, 625, 313, 156, 78.1, and 39.1 pg/mL) and diluted sample were added separately to 96-well plate wells containing goat anti-mouse IgG, and duplicate experiments were carried out. At the same time, the assay buffers (150 and 100 μL) were added to four other plate wells for blank and TA treatments. With the exception of blank and TA, 50 μL of alkaline phosphatase PGE_2 conjugate was added to each cell, followed by the addition of 50 μL of PGE_2 EIA antibody to B_0, standards, and samples. After incubation at room temperature for 2 h, all plates were washed with washing buffer to remove residual reagent. For TA treatment, a 5 μL alkaline phosphatase PGE_2 conjugate was added followed by 200 μL of *p*-nitrophenyl phosphate (pNpp) substrate. After incubation at room temperature for 45 min, a 50 μL stop solution was added to terminate the reaction, and the absorbance was measured at 405 nm. The concentration of PGE_2 was calculated on the basis of the linear regression equations of the standard curve from percent bound (% B/B_0) using the following formula:

$$\%\ B/B_0 = (\text{absorbance of standard or sample} - \text{absorbance of blank} - \text{absorbance of NSB})/(\text{absorbance of } B_0 - \text{absorbance of blank} - \text{absorbance of NSB}) \times 100\%$$

Determination of Nitric Oxide. One hundred microliters of seven concentrations of $NaNO_2$ standard (100, 80, 60, 40, 20, 10, and 5 μM) and samples (peritoneal exudate cell supernatant and peritoneal exudate fluid) were added separately to the 96-well plate wells, with 5% phosphoric acid solution as blank treatment. Next, 100 μL of Griess reagent was added to each well for 5 min, and the absorbance was measured at 540 nm with an ELISA reader. The Griess reagent was freshly prepared by mixing 30 mL of reagent A (1% sulfanilamide in 5% phosphoric acid solution) and 30 mL of reagent B (0.1% *N*-1-naphthylethylenediamide dihydrochloride solution). The level of nitric oxide was calculated on the basis of the linear regression equation of the standard curve.

Proliferation of Splenocyte. One hundred microliters of splenocyte with a density of 4×10^6 cells/mL was seeded to a 96-well plate well, followed by the addition of 100 μL of LPS (10 μg/mL) and 100 μL of Con A (5 μg/mL) to stimulate proliferation of B and T cells, respectively. Additionally, 100 μL of CM-10 culture medium was used as blank treatment. All of these treatments were conducted in triplicate. After incubation in an incubator (5% CO_2) at 37 °C for 48 h, 20 μL of MTT reagent (5 μg/mL) was added to each well and further incubated in the dark for another 4 h. Then the solution was centrifuged at 2400 rpm (4 °C) for 10 min. The supernatant was discarded, and 200 μL of DMSO was added to dissolve the violet crystal; the absorbance was measured at 570 nm. The data were expressed as stimulating index (SI) using the following formula:

$$\text{SI} = (\text{absorbance after stimulation with LPS or Con A} - \text{absorbance of CM-10 culture medium})/(\text{absorbance without stimulation} - \text{absorbance of CM-10 culture medium})$$

Statistical Analysis. All of the data were subjected to analysis of variance using SAS (*17*) and Duncan's multiple-range test for significance comparison ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Effect of Roasting on Acetylglucoside and Aglycone Contents in Soybean Cake. Table 1 shows the effect of roasting on acetylglucoside and aglycone contents (micrograms per gram) in soybean cake. After roasting at 200 °C for 10 min, the levels of acetylglucoside and aglycone rose by 294.2 and 609.2 μg/g, respectively. However, with a temperature 200 °C and a heating time 30 min, no acetylglucoside was detected, whereas the amount of aglycone further increased to 975.6 μg/g. This outcome demonstrated that with prolonged heating time at high temperature, a complete conversion from acetylglucoside to its corresponding aglycone is possible. The same phenomenon was observed by Chien et al. (*16*).

Table 1. Effect of Roasting on Acetylglucoside and Aglycone Isoflavone Contents (Micrograms per Gram)[a] in Soybean Cake

		after roasting	
isoflavone	before roasting	(200 °C, 10 min)	(200 °C, 30 min)
acetyldaidzin	90.3 ± 5.0	186.6 ± 0.6	nd[b]
acetylglycitin	88.1 ± 0.6	134.5 ± 1.8	nd
acetylgenistin	141.1 ± 4.6	292.1 ± 6.1	nd
subtotal	319.0 ± 4.8	613.2 ± 5.0	nd
daidzein	78.4 ± 1.2	285.2 ± 2.4	438.3 ± 2.4
glycitein	40.1 ± 0.3	273.8 ± 2.0	193.8 ± 8.3
genistein	110.1 ± 3.0	279.9 ± 2.9	343.5 ± 6.2
subtotal	228.6 ± 2.1	837.8 ± 1.4	975.6 ± 0.3

[a] Average of duplicate analyses ± standard deviation. [b] Not detected.

Functional Components in Isoflavone Powder. Table 2 shows contents of functional components in isoflavone powder. Similar to a report by Kao and Chen (*3*), the acetylglucoside powder was found to contain the highest levels of soyasapogenol A (2.4 mg/g) and soyasapogenol B (24.3 mg/g), followed by ISO-2 (0.8 and 6.1 mg/g), ISO-1 (1.7 and 2.9 mg/g), glucoside (0.8 and 1.2 mg/g), and aglycone (1.3 mg/g soyasapogenol B), whereas no saponin was detected in the malonylglucoside powder. However, compared to the other powder, the aglycone powder showed a larger amount of total flavonoids and phenolic compounds, which equaled 3.8 and 15.6 mg/g, respectively. Significant amounts of total flavonoids and total phenolic compounds were also present in the acetylglucoside powder (3.3 and 10.3 mg/g) and ISO-2 (2.0 and 12.6 mg/g), whereas both glucoside and malonylglucoside powders contained a low level. This result suggested that the acetylglucoside powder may show a higher biological activity than the other powders. No phenolic acid, α-tocopherol, and ascorbic acid were detected in any of the isoflavone powders.

Effect of Isoflavone Powder Extract on Body Weight of BALB/c Mice. Table 3 shows the effect of isoflavone powder extract on the body weight of BALB/c mice during feeding for 4 weeks. A slight increase in body weight was found for all 11 treatments, but there was no significant difference among most treatments over a 4 week feeding period. This result is similar to a report by Paradkar et al. (*13*), who observed no significant difference in the body weight of C57/BL6 mice fed a Novasoy diet containing 0.3% isoflavone.

Effect of Isoflavone Powder Extract on Leukocyte Number of BALB/c Mice. Figure 1 shows the effect of isoflavone powder extract on the leukocyte number of BALB/c mice stimulated by LPS. Of the various treatments, the control and γ-PGA treatments showed high leukocyte densities at 9.6×10^6/mL and 5.3×10^6/mL, respectively, whereas a low density (1.3×10^6/mL) occurred for the normal treatment without LPS stimulation. Compared to PDTC treatment, all of the isoflavone treatments showed no significant difference in leukocyte number. This result implied that isoflavone powders were effective in inhibiting proliferation of leukocyte in the blood of BALB/c mice to alleviate inflammation and is comparable to PDTC drug treatment. It has been well established that acute inflammation can result in proliferation of leukocyte and formation of C-reactive protein (*2*, *8*).

Effect of Isoflavone Powder on Inflammation Mediator Formation. Cytokines including IL-1β, IL-6, and TNF-α were investigated in our study. However, only the results of IL-1β

Table 2. Contents[a] (Milligrams per Gram) of Functional Components in Isoflavone Powders[b]

isoflavone powder	phenolic acids			total flavonoids[c]	total phenolic compounds[d]	α-tocopherol	ascorbic acid	saponins	
	gallic acid	caffeic acid	chlorogenic acid					soyasapogenol A	soyasapogenol B
M	nd[e]	nd	nd	0.5 ± 0.0e	6.8 ± 0.3d	nd	nd	nd	nd
G	nd	nd	nd	0.4 ± 0.0f	6.1 ± 0.1e	nd	nd	0.8 ± 0.0c	1.2 ± 0.0d
Ac	nd	nd	nd	3.3 ± 0.1b	10.3 ± 0.2c	nd	nd	2.4 ± 0.1a	24.3 ± 2.1a
Ag	nd	nd	nd	3.8 ± 0.1a	15.6 ± 1.0a	nd	nd	nd	1.3 ± 0.0e
ISO-1	nd	nd	nd	1.0 ± 0.0d	10.5 ± 0.1c	nd	nd	1.7 ± 0.0b	2.9 ± 0.1c
ISO-2	nd	nd	nd	2.0 ± 0.0c	12.6 ± 0.4b	nd	nd	0.8 ± 0.0c	6.1 ± 0.5b

[a] Average of duplicate analyses ± standard deviation. [b] Symbols bearing different letters (a−e) in the same column are significantly different ($P < 0.05$). [c] Data expressed as μg/mL of catechin equivalents. [d] Data expressed as μg/mL of gallic acid equivalents. [e] Not detected. M, malonylglucoside powder; G, glucoside powder; Ac, acetylglucoside powder; Ag, aglycone powder; ISO-1, powder made with soybean cake extract containing 12 isoflavones; ISO-2, powder made with a combination of four groups of isoflavone extract.

Table 3. Effects of Isoflavone Powders on Body Weight of BALB/c Mice[a]

treatment	week				
	0	1	2	3	4
M	20.8 ± 1.2 a	21.8 ± 0.6 ab	22.1 ± 0.6 a	21.1 ± 1.0 bc	22.2 ± 0.9 a
G	20.8 ± 1.1 a	22.2 ± 0.9 ab	22.8 ± 1.0 a	22.4 ± 1.2 abc	22.7 ± 0.9 a
Ac	20.8 ± 1.1 a	21.9 ± 1.0 ab	22.2 ± 1.2 a	21.3 ± 0.7 bc	22.0 ± 1.2 a
Ag	20.8 ± 1.1 a	22.1 ± 1.5 ab	22.0 ± 1.0 a	21.7 ± 0.9 abc	22.1 ± 1.0 a
ISO-1	20.7 ± 1.1 a	21.4 ± 0.8 ab	22.0 ± 1.0 a	21.5 ± 1.0 abc	22.1 ± 1.0 a
ISO-2	20.6 ± 0.8 a	21.4 ± 1.1 ab	21.6 ± 1.1 a	21.2 ± 0.6 c	21.8 ± 0.7 a
Gein	20.8 ± 1.1 a	22.0 ± 1.2 ab	22.8 ± 1.3 a	21.4 ± 1.0 abc	22.0 ± 0.7 a
γ-PGA	20.9 ± 1.2 a	20.2 ± 1.4 b	21.5 ± 0.7 a	21.4 ± 1.0 abc	21.6 ± 0.9 a
Ctrl	20.9 ± 1.3 a	22.7 ± 0.8 a	23.0 ± 1.1 a	22.5 ± 1.0 abc	22.8 ± 0.9 a
Nor	21.1 ± 1.2 a	23.2 ± 1.3 a	23.1 ± 1.1 a	22.8 ± 0.6 a	23.1 ± 1.0 a
PDTC	21.2 ± 1.2 a	22.4 ± 0.9 ab	22.9 ± 1.0 a	22.7 ± 0.8 ab	23.1 ± 1.3 a

[a] Symbols bearing different letters (a−c) in the same column are significantly different ($P < 0.05$). M, malonylglucoside isoflavone powder; G, glucoside isoflavone powder; Ac, acetylglucoside isoflavone powder; Ag, aglycone isoflavone powder; ISO-1, powder from soybean cake extract containing 12 isoflavones; ISO-2, powder from a combination of four groups of isoflavone extracts; Gein, genistein; γ-PGA, poly(γ-glutamic acid); Ctrl, control; Nor, normal; PDTC, ammonium pyrrolidinedithiocarbamate.

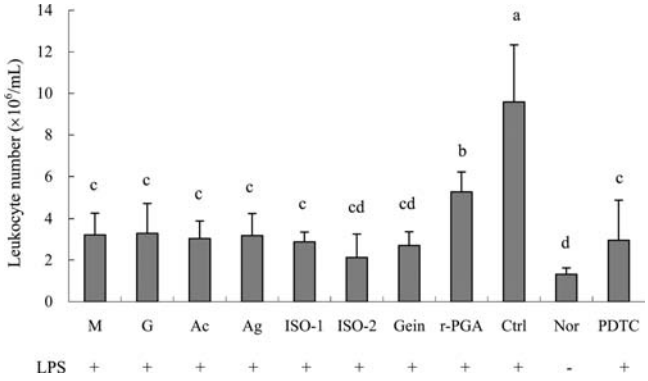

Figure 1. Effects of isoflavone powders on leukocyte number of BALB/c mice. M, malonylglucoside isoflavone powder; G, glucoside isoflavone powder; Ac, acetylglucoside isoflavone powder; Ag, aglycone isoflavone powder; ISO-1, powder from soybean cake extract containing 12 isoflavones; ISO-2, powder from a combination of four groups of isoflavone extracts; Gein, genistein; γ-PGA, poly(γ-glutamic acid); Ctrl, control; Nor, normal; PDTC, ammonium pyrrolidinedithiocarbamate.

and IL-6 are shown as no TNF-α was detected. **Figure 2A** shows the effect of isoflavone powder on IL-1β secretion of cultured peritoneal exudate cell without LPS stimulation. The highest amount of IL-1β (671 pg/mL) was shown for the control treatment, followed by PDTC (388 pg/mL), γ-PGA (370 pg/mL), acetylglucoside (312 pg/mL), genistein (280 pg/mL), glucoside (266 pg/mL), ISO-1 (258 pg/mL), malonylglucoside (254 pg/mL), ISO-2 (203 pg/mL), aglycone (196 pg/mL), and the normal treatment (65 pg/mL). Conversely, a higher level of IL-1β was produced for cultured peritoneal exudate cell stimulated with LPS (**Figure 2B**), but a similar trend was followed: control treatment (1087 pg/mL) > γ-PGA (641 pg/mL) > PDTC (488 pg/mL) > genistein (482 pg/mL) > acetylglucoside (462 pg/mL) > malonylglucoside (443 pg/mL) > ISO-1 (435 pg/mL) > glucoside (432 pg/mL) > ISO-2 (376 pg/mL) > aglycone (368 pg/mL) > normal treatment (291 pg/mL). However, for peritoneal exudate fluid secretion (**Figure 2C**), both γ-PGA and control treatments showed a high level, which amounted to 585 and 570 pg/mL, respectively, whereas a significantly lower level occurred for aglycone (431 pg/mL), genistein (409 pg/mL), and the normal treatment (91 pg/mL), and there were no significant differences between any of the other six treatments.

Figure 3A shows the effect of isoflavone powder on IL-6 secretion of cultured peritoneal exudate cell without LPS stimulation. Similar to IL-1β, a high level of IL-6 was shown for the control and γ-PGA treatments, which equaled 4.13 and 2.20 pg/mL, respectively, whereas the other treatments showed no significant difference. It is worth pointing out that compared to the normal treatment (0.64 ng/mL), a lower concentration was found for acetylglucoside (0.57 ng/mL), ISO-1 (0.51 ng/mL), PDTC (0.46 ng/mL), aglycone (0.44 ng/mL), malonylglucoside (0.37 ng/mL), and glucoside (0.36 ng/mL). After LPS stimulation, a pronounced increase of IL-6 was shown for all treatments (**Figure 3B**). Likewise, both control and γ-PGA treatments showed a marked rise to 17.90 and 16.97 ng/mL, respectively, with a much lower level occurring for the other treatments, including ISO-1 (6.55 ng/mL), glucoside (6.37 ng/mL), normal treatment (6.24 ng/mL), acetylglucoside (5.63 ng/mL), malonylglucoside (5.21 ng/mL), aglycone (5.14 ng/mL), ISO-2 (4.80 ng/mL), genistein (4.20 ng/mL), and PDTC treatment (3.25 ng/mL).

For peritoneal exudate fluid, the control treatment showed the largest IL-6 secretion (6.69 ng/mL), followed by γ-PGA (3.66 ng/mL) and malonylglucoside (2.85 ng/mL), and there

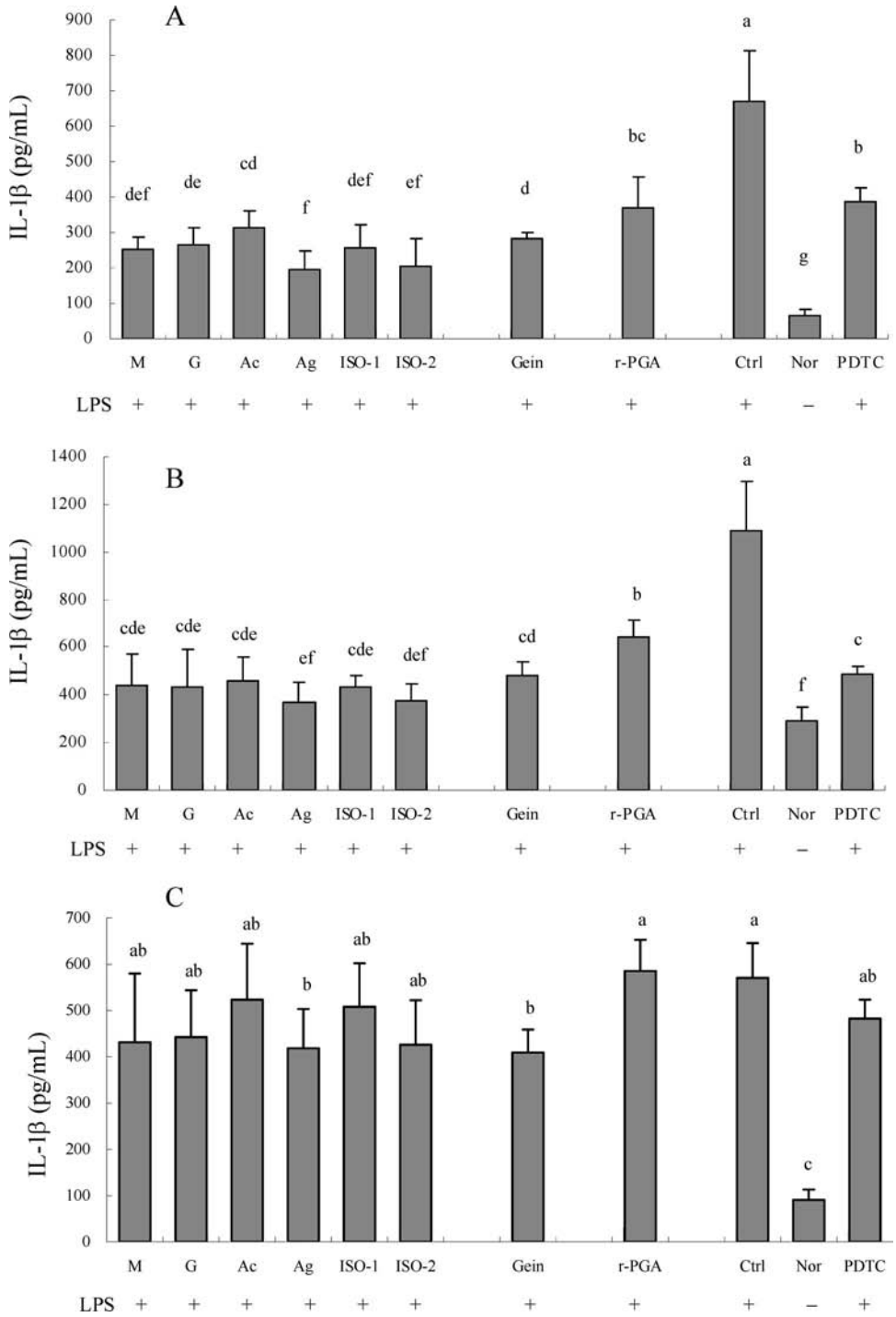

Figure 2. Effects of isoflavone powders on IL-1β secretion of cultured peritoneal exudate cell only (**A**) and cultured peritoneal exudate cell stimulated with LPS (10 μg/mL) for 24 h (**B**) as well as peritoneal exudate fluid (**C**) for BALB/c mice. M, malonylglucoside isoflavone powder; G, glucoside isoflavone powder; Ac, acetylglucoside isoflavone powder; Ag, aglycone isoflavone powder; ISO-1, powder from soybean cake extract containing 12 isoflavones; ISO-2, powder from a combination of four groups of isoflavone extracts; Gein, genistein; γ-PGA, poly(γ-glutamic acid); Ctrl, control; Nor, normal; PDTC, ammonium pyrrolidinedithiocarbamate.

were no significant differences between any of the other treatments (**Figure 3C**), that is, acetylglucoside (1.95 ng/mL), glucoside (1.85 ng/mL), ISO-2 (1.75 ng/mL), genistein (1.60 ng/mL), ISO-1 (1.51 ng/mL), PDTC (1.39 ng/mL), normal (1.37 ng/mL), and aglycone treatment (1.36 ng/mL).

Panels **A** and **B** of **Figure 4** show the effect of isoflavone powder on nitric oxide secretion of cultured peritoneal exudate cell without and with LPS stimulation, respectively. Without LPS stimulation, with the exception of aglycone, all of the other treatments were significantly lower in NO level than the control treatment (22.9 μM). Concentrations of 11.2, 10.5, 9.4, 9.3, 9.2, 8.7, 6.5, 6.0, and 5.1 μM NO were generated for aglycone, ISO-2, malonylglucoside, PDTC, genistein, glucoside, ISO-1, acetylglucoside, and γ-PGA treatment, whereas a minor amount was formed for the normal treatment. After LPS and IFN-γ stimulation for 24 h, the NO secretion was significantly higher than those without LPS and IFN-γ stimulation. Similarly, the control treatment showed the highest NO level (57.2 μM), followed by

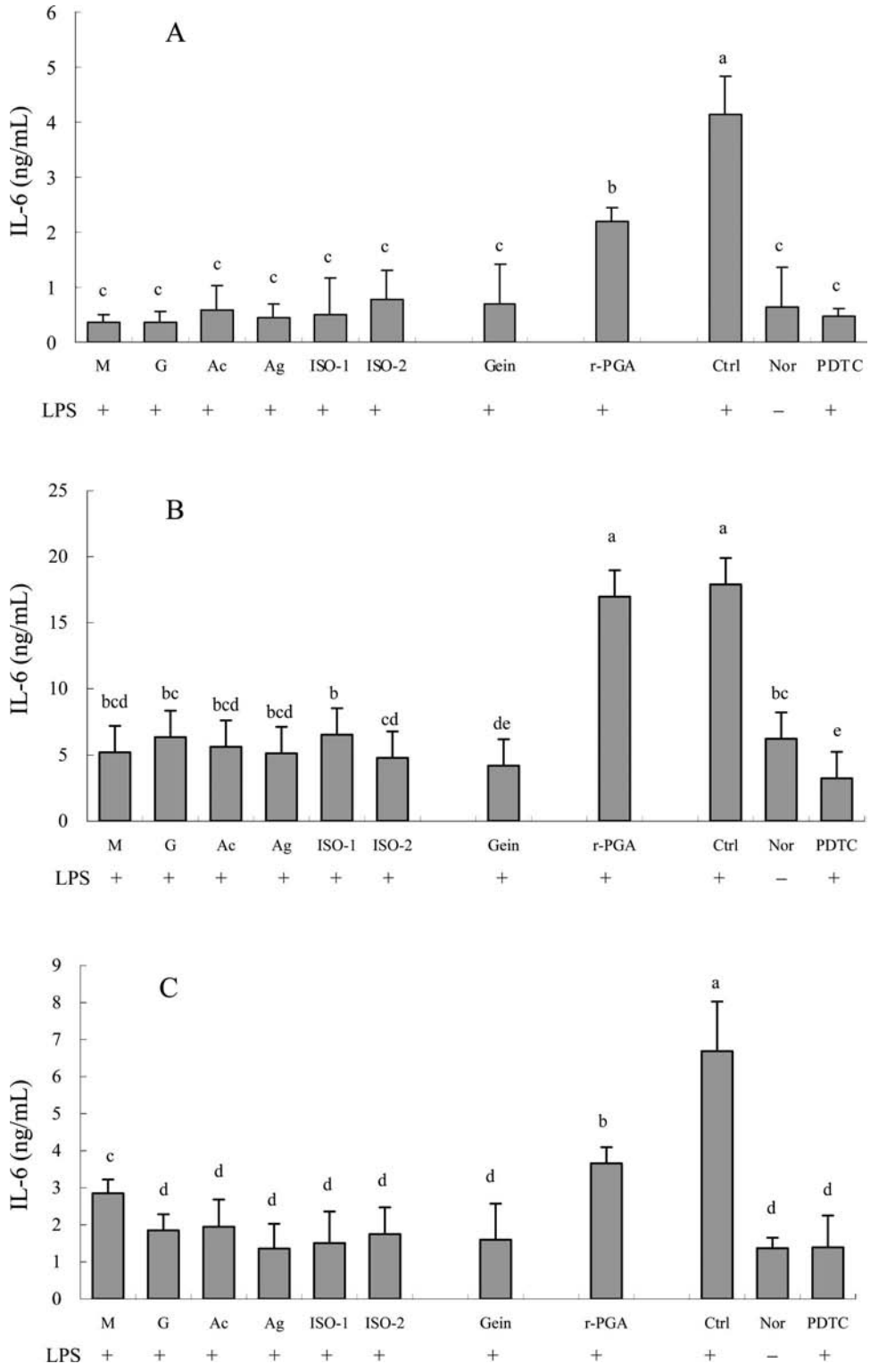

Figure 3. Effects of isoflavone powders on IL-6 secretion of cultured peritoneal exudate cell only (**A**) and cultured peritoneal exudate cell stimulated with LPS (10 μg/mL) for 24 h (**B**) as well as peritoneal exudate fluid (**C**) for BALB/c mice. M, malonylglucoside isoflavone powder; G, glucoside isoflavone powder; Ac, acetylglucoside isoflavone powder; Ag, aglycone isoflavone powder; ISO-1, powder from soybean cake extract containing 12 isoflavones; ISO-2, powder from a combination of four groups of isoflavone extracts; Gein, genistein; γ-PGA, poly(γ-glutamic acid); Ctrl, control; Nor, normal; PDTC, ammonium pyrrolidinedithiocarbamate.

glucoside (28.8 μM), PDTC (21.9 μM), ISO-1 (21.1 μM), acetylglucoside (20.9 μM), γ-PGA (20.5 μM), normal (19.5 μM), genistein (18.7 μM), malonylglucoside (18.5 μM), ISO-2 (15.0 μM), and aglycone (13.7 μM). For peritoneal exudate fluid, only four treatments of glucoside, acetylglucoside, ISO-2, and normal were significantly lower than the control treatment, with the other treatments showing no significant difference (**Figure 4C**). The amount of NO secreted followed the order ISO-1 (16.2 μM), malonylglucoside (14.3 μM), genistein (13.7 μM), γ-PGA (12.2 μM), control (11.3 μM), PDTC (9.6 μM), glucoside (6.6 μM), aglycone (6.5 μM), ISO-2 (6.1 μM), acetylglucoside (5.3 μM), and normal (trace).

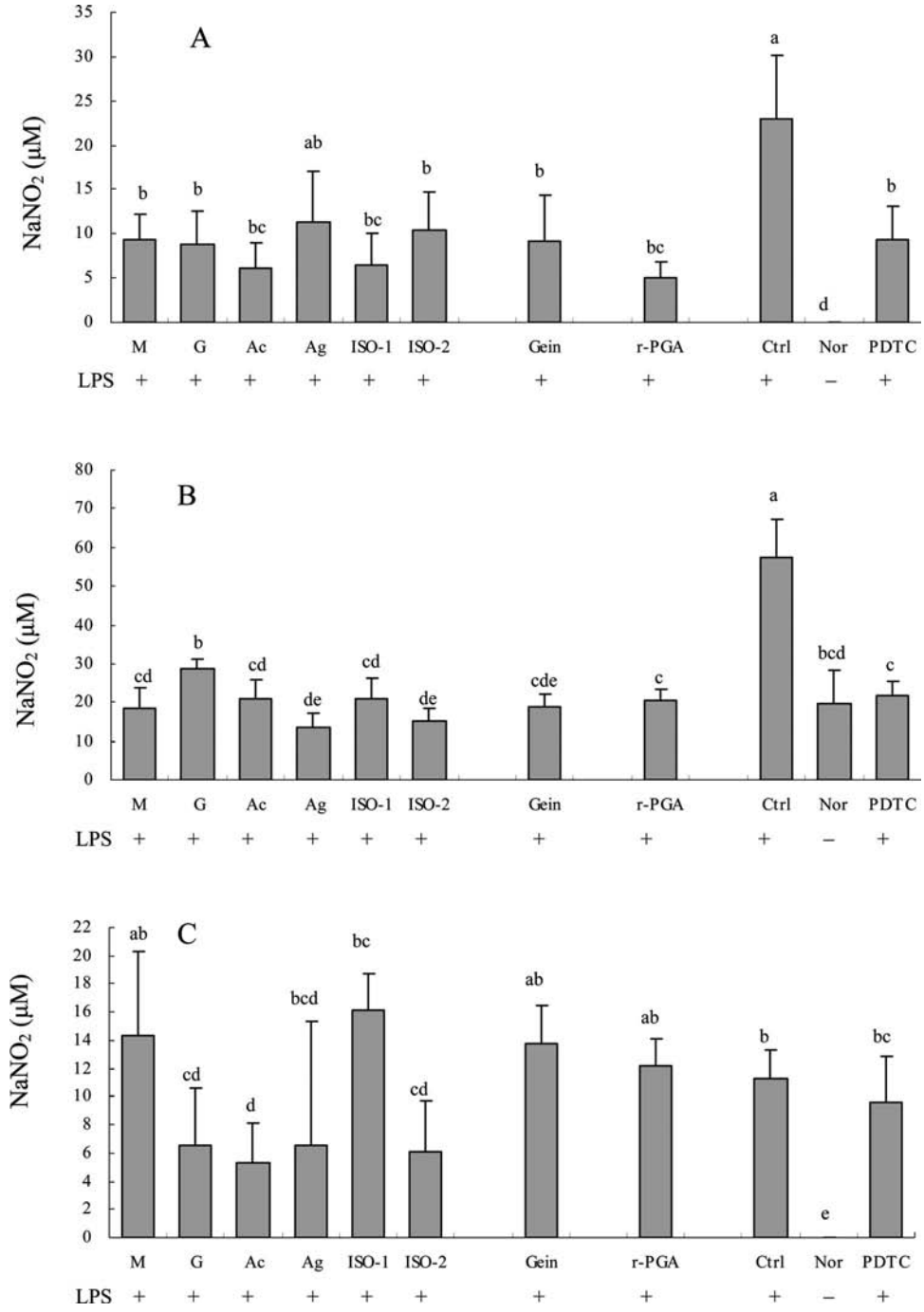

Figure 4. Effects of isoflavone powders on nitric oxide secretion of cultured peritoneal exudate cell only (**A**) and cultured peritoneal exudate cell stimulated with LPS (50 ng/mL) and IFN-γ (25 unit/mL) for 24 h (**B**) as well as peritoneal exudate fluid (**C**) for BALB/c mice. M, malonylglucoside isoflavone powder; G, glucoside isoflavone powder; Ac, acetylglucoside isoflavone powder; Ag, aglycone isoflavone powder; ISO-1, powder from soybean cake extract containing 12 isoflavones; ISO-2, powder from a combination of four groups of isoflavone extracts; Gein, genistein; γ-PGA, poly(γ-glutamic acid); Ctrl, control; Nor, normal; PDTC, ammonium pyrrolidinedithiocarbamate.

Panels **A** and **B** of **Figure 5** show the effect of isoflavone powder on PGE_2 secretion of cultured peritoneal exudate cell without and with LPS stimulation, respectively. Without LPS stimulation, the control treatment showed the highest level of PGE_2 (2.42 ng/mL), followed by PDTC treatment (1.37 ng/mL), γ-PGA (0.98 ng/mL), malonylglucoside (0.76 ng/mL), genistein (0.76 ng/mL), ISO-1 (0.74 ng/mL), aglycone (0.64 ng/mL), ISO-2 (0.60 ng/mL), normal (0.55 ng/mL), acetylglucoside (0.55 ng/mL), and glucoside (0.51 ng/mL). All of the isoflavone powders showed a significantly lower secretion of PGE_2 than the control treatment, but there were no significant differences between any of these isoflavone treatments. In addition, both genistein and γ-PGA were effective in inhibiting PGE_2 formation. After LPS stimulation, the amounts of PGE_2 formed were significantly higher than those without LPS stimulation. However, a similar tendency was followed with the control treatment showing the highest level (20.36 ng/mL), followed by normal (18.45 ng/mL), γ-PGA (17.51 ng/mL), ISO-1 (13.01 ng/mL), glucoside (12.60 ng/mL), aglycone (12.29 ng/mL), acetylglucoside (11.42 ng/mL), genistein (11.00 ng/mL), ISO-2 (10.30 ng/mL), malonylglucoside (9.04 ng/mL), and PDTC (7.21 ng/mL). With the exception of γ-PGA and normal treatment, the levels of PGE_2 secreted by the other treatments were

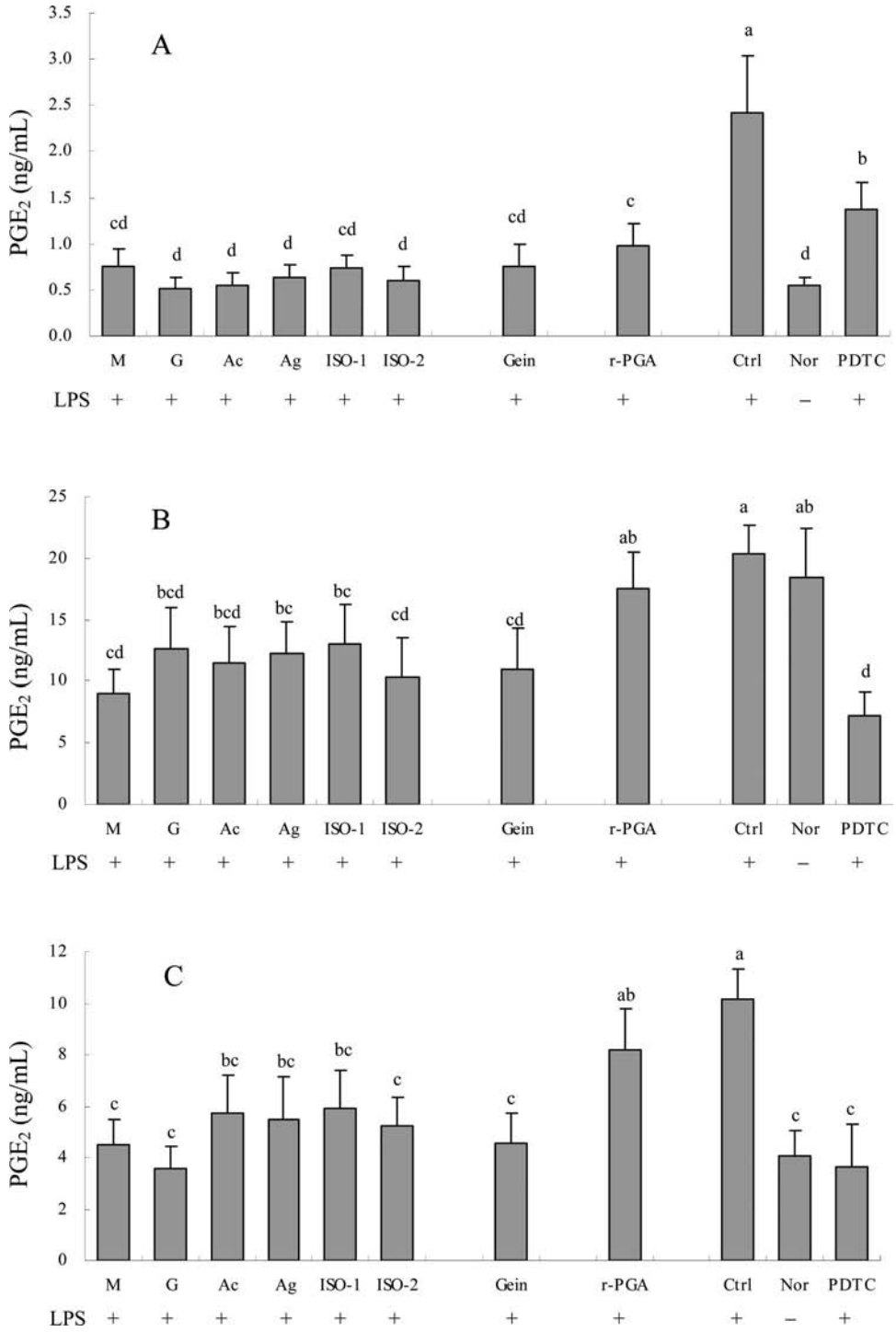

Figure 5. Effects of isoflavone powders on PGE_2 secretion of cultured peritoneal exudate cell only (**A**) and cultured peritoneal exudate cell stimulated with LPS (10 μg/mL) for 24 h (**B**) as well as peritoneal exudate fluid (**C**) for BALB/c mice. M, malonylglucoside isoflavone powder; G, glucoside isoflavone powder; Ac, acetylglucoside isoflavone powder; Ag, aglycone isoflavone powder; ISO-1, powder from soybean cake extract containing 12 isoflavones; ISO-2, powder from a combination of four groups of isoflavone extracts; Gein, genistein; γ-PGA, poly(γ-glutamic acid); Ctrl, control; Nor, normal; PDTC, ammonium pyrrolidinedithiocarbamate.

lower than that with the control treatment, indicating γ-PGA failed to inhibit PGE_2 formation as stimulated by LPS. A high amount of PGE_2 generated for the control treatment is probably due to excessive reaction of the cell to LPS stimulation. In addition to aglycone and ISO-1 treatments, there were no significant differences between any of the isoflavone treatments.

Figure 5C shows the effect of isoflavone powder on PGE_2 secretion of peritoneal exudate fluid of BALB/c mice. Likewise, with the exception of γ-PGA, the amounts of PGE_2 produced by the other treatments were significantly lower than the control treatment and followed the order control (10.18 ng/mL) > γ-PGA (8.20 ng/mL) > ISO-1 (5.88 ng/mL) > acetylglucoside (5.71 ng/mL) > aglycone (5.48 ng/mL) > ISO-2 (5.20 ng/mL) > genistein (4.56 ng/mL) > malonylglucoside (4.52 ng/mL) > normal (4.08 ng/mL) > PDTC (3.61 ng/mL) > glucoside (3.55 ng/mL). This result implied that all of the isoflavone powder treatments were as effective in inhibiting PGE_2 formation as both genistein and PDTC

Table 4. Effects of Isoflavone Powders on Proliferation of Splenocytes for BALB/c Mice[a]

treatment	stimulating index	
	cell + Con A (2.5 μg/mL)	cell + LPS (5 μg/mL)
M	7.8 ± 0.7 abc	6.2 ± 0.4 a
G	7.1 ± 0.3 c	5.3 ± 0.8 ab
Ac	7.6 ± 0.2 bc	4.0 ± 0.5 cde
Ag	7.5 ± 0.6 bc	4.2 ± 0.4 cd
ISO-1	7.7 ± 0.3 abc	3.2 ± 0.6 e
ISO-2	7.9 ± 0.9 abc	3.3 ± 0.3 e
Gein	7.3 ± 0.6 bc	3.4 ± 1.0 cde
γ-PGA	7.7 ± 0.7 abc	5.3 ± 1.2 ab
Ctrl	9.2 ± 1.0 a	5.1 ± 1.2 abc
Nor	8.8 ± 0.7 a	5.5 ± 0.8 abc
PDTC	8.3 ± 0.7 ab	4.4 ± 1.0 bc

[a] Symbols bearing different letters (a−e) in the same column are significantly different ($P < 0.05$). M, malonylglucoside isoflavone powder; G, glucoside isoflavone powder; Ac, acetylglucoside isoflavone powder; Ag, aglycone isoflavone powder; ISO-1, powder from soybean cake extract containing 12 isoflavones; ISO-2, powder from a combination of four groups of isoflavone extracts; Gein, genistein; γ-PGA, poly(γ-glutamic acid); Ctrl, control; Nor, normal; PDTC, ammonium pyrrolidinedithiocarbamate.

treatments, and there were no significant differences between any of these isoflavone powder treatments.

Table 4 shows the effect of isoflavone powders on proliferation of splenocytes of BALB/c mice. For T cell proliferation, a slightly higher stimulating index was found for both control and normal treatments after Con A stimulation, whereas no significant differences occurred among the other treatments. Compared to the control treatment, the isoflavone treatments including glucoside, acetylglucoside, aglycone, and genistein powders showed a lower stimulating index, whereas the other treatments showed no significant difference. However, when compared to genistein and γ-PGA, there were no significant differences for all of the isoflavone powders, revealing the isoflavone powder and γ-PGA failed to inhibit T cell proliferation. The stimulating index of all the treatments after Con A stimulation followed the order control (9.2) > normal (8.8) > PDTC (8.3) > ISO-2 (7.9) > malonylglucoside (7.8) > ISO-1 (7.7) = γ-PGA (7.7) > acetylglucoside (7.6) > aglycone (7.5) > genistein (7.3) > glucoside (7.1). Conversely, a different trend was observed for B cell proliferation stimulated by LPS, and the highest stimulating index was shown for malonylglucoside (6.2), followed by normal (5.5), γ-PGA (5.3), glucoside (5.3), control (5.1), PDTC (4.4), aglycone (4.2), acetylglucoside (4.0), genistein (3.4), ISO-2 (3.3), and ISO-1 (3.2). With the exception of ISO-2 and ISO-1, there were no significant differences in stimulating index between control treatment and the other isoflavone treatments, demonstrating both ISO-1 and ISO-2 could retard B cell proliferation.

In this study the inflammation was induced by intraperitoneal injection of LPS, a major component of the cell wall of Gram-negative bacteria. LPS is mainly composed of lipid A and polysaccharide, with the former being toxic to eukaryotic cell (*18*). The major cell for immunity in the abdominal cavity is mainly the macrophage, playing an important role for innate immunity and adaptive immunity. It has been well documented that macrophages can be activated by cytokines such as interferon-γ or microorganism components such as lipopolysaccharide (*19*). In innate immunity, the macrophage can kill bacteria and release inflammation mediators such as nitric oxide, but for adaptive immunity, the macrophage belongs to antigen-presenting cells and secretes cytokines such as TNF-α, IL-6, and IL-12 to regulate the function of helper T cells (*20*). In our study we used LPS to induce inflammation for BALB/c mice and then activate intraperitoneal macrophages for secretion of inflammation mediators and proliferation of macrophages, both of which can be inhibited in the presence of anti-inflammatory functional components such as isoflavone extracts from soybean cake. In addition, in this study we also used PDTC drug for comparison. PDTC is an inhibitor of nuclear factor-κB (NF-κB), which can increase gene expression of inflammation mediators such as IL-6, IL-12, and IL-8, as well as adhesion factors such as E-selectin. Also, PDTC can regulate the formation of TNF-α and IL-1β, both of which can interact with each other to activate NF-$\kappa\beta$ and in turn cause inflammation to proceed (*21*).

Nitric oxide is a kind of free radical that can penetrate cell membranes to translate biochemical signals (*22*). Nitric oxide can be synthesized by nitric oxide synthase (NOS) and has been shown to contain three isomers, nerunal NOS (nNOS or NOS I), endothelial NOS (eNOS or NOS III), and inducible NOS (iNOS or NOS II). Nerunal NOS (molecular mass = 155 kDa) is mainly present in neurons, skeletal muscle fiber, and lung epithelial tissue (*23*), whereas endothelial NOS (molecular mass = 140 kDa) is present in endothelial tissue and neurons as well as cardiac muscle cell (*24*). Both eNOS and nNOS are also named as constitutive NOS because of their continued expression in fiber cells, with their activities being regulated by calcium ion concentration and involved in signal transduction, vasodilation, pressure regulation, and platelet antiagglutination (*22, 25*). However, iNOS (molecular mass = 130 kDa) is present in macrophages, monocytes, neutrophils, endothelium cells, and smooth muscle cells and can result in the formation of a large amount of NOS, which is 1000 times higher than constitutive NOS when stimulated by LPS or cytokine (*26, 27*). A high level of NOS has been shown to activate leukocyte to induce inflammation, resulting in damage of blood vessel endothelium and oxidation of low-density lipoprotein to promote platelet agglutination and atherosclerosis through reaction with superoxide anion to form peroxynitrite (*27*). In addition, an excessive level of NOS has been demonstrated to induce cancer through damage to the DNA sequence and production of oxidative stress (*28*).

In our experiment we proved that both isoflavone powder from soybean cake and genistein standard are effective in retarding inflammation, lowering the number of leukocytes in mouse blood, and decreasing secretion of inflammation mediators such as IL-1β, IL-6, NO, and PGE_2 in both peritoneal exudate cell and peritoneal exudate fluid for BALB/c mice, as well as reducing proliferation of B cells. Most studies in the literature are focused on the effect of isoflavones on chronic inflammation in patients or in vitro study. Paradkar et al. (*13*) reported that with levels of 2, 10, 30 and 100 μM genistein, the secretion of IL-6 by Caco-2 cells was retarded, and the higher the level of genistein, the better the inhibition effect. Also, a concentration of 30 μM biochanin A, kaempferol, quercetin, and epigallocatechin showed the same effect. In another study Chacko et al. (*14*) indicated that genistein may activate PPAR-γ for inhibition of monocyte adhesion to human vascular endothelial cells. In a similar report Hall et al. (*2*) showed that soybean products rich in isoflavone may raise the level of C-reactive protein, but was ineffective in terms of cell adhesion factor. In a study dealing with the anti-inflammation of microglia cells as affected by isoflavone extracts from *Pueraria thunbergiana*, Park et al. (*7*) concluded that the aglycones were more efficient in inhibiting the secretion of NO, TNF-α, and IL-1β than their corresponding glucosides. Similarly, Takano-Ishikawa et al. (*29*)

revealed that both genistein and daidzein were effective in retarding PGE_2 production from macrophages stimulated by LPS, with the former being more pronounced than the latter, probably because of the presence of one more hydroxyl group for genistein. It has been inferred that genistein may activate PPAR-γ, a transcription factor present in adipose tissue and the adrenal gland, spleen, large colon, and immune system, which in turn reduces pro-inflammatory gene expression and retards tumor cell proliferation (*5*, *30*, *31*). In addition, both genistein and daidzein have been shown to inhibit the activity of cyclooxygenase-2 (COX-2), a major enzyme causing PGE_2 formation via catalysis of arachidonic acid (*29*). Several reports also indicated that many flavonoids possess the ability to lower iNOS protein expression so that the formation of NO can be retarded (*32*).

In addition to macrophage, the splenocyte also plays a vital role in inflammation because of the presence of T and B cells. Our result showed that both ISO-1 and ISO-2 could inhibit B cell proliferation in spleen stimulated by LPS, implying that the isoflavone powder may be toxic to B cells. Nevertheless, B cells are mainly responsible for antibody formation, in which IgE can bind with cells containing Fc receptors such as FcϵRI or FcϵRII even in the absence of antigen (*8*). The Fc receptors are mainly present in mast cells, monocytes, basophils, and eosinophils. Mast cells have been shown to contain numerous large granules, in which inflammation mediators such as histamine and leukotriene can bind with IgE to form complexes prior to activation. However, the mediators can be released after activation of the mast cell through binding of antigen and IgE, which in turn resulted in vasodilation, allergy, and asthma (*8*, *33*). Nontheless, the reduction of IgE formation as affected by isoflavone powder needs further investigation.

We have to point out here that there were no significant differences in anti-inflammatorty activity between any of the isoflavone powder treatments, which may be associated with metabolism. It has been well established that the glucose-containing isoflavones can be hydrolyzed by β-glucosidase in the intestine to form aglycone for absorption, after which most aglycones can enter into the first hepatic metabolism route for conjugation with glucuronic acid, whereas a small portion was catalyzed to form sulfate derivative by sulfotransferase (*34*). The results of several in vitro studies have indicated that glucosides were inferior to aglycones in anti-inflammatory activity (*7*, *29*), but the sugar moieties in isoflavones in those experiments were different from ours, as shown above. In a study dealing with the effect of morin and morin sulfate/glucuronide on LPS-stimulated mouse macrophage cell (RAW 264.7) and the formation of inflammation mediators in peritoneal exudate cell of mice, Fang et al. (*20*) reported that with morin and morin sulfate/glucuronide levels at 1.25 mM and 1.25 μM, respectively, a 50% inhibition of NO from macrophage RAW 264.7 was observed. Likewise, the concentrations required for inhibition of 50% NO from peritoneal exudate cell were 1.5 mM and 1.5 μM for morin and morin sulfate/glucuronide, respectively. This phenomenon revealed that the glucuronide-containing derivative formed from morin during metabolism was 1000 times more effective in anti-inflammatory activity than morin. In our experiment the various treatments containing different chemical forms of isoflavone glucosides were subjected to tube feeding, but the differences among all of the treatments were minimal as isoflavone glucosides should be converted into isoflavone glucuronides and sulfates in vivo.

In addition to isoflavones, the presence of some other functional components may also contribute to the anti-inflammatory activity, especially for the aglycone and acetylglucoside powders, with the former containing a high level of total phenolic compounds and total flavonoids and the latter a high amount of saponins. Although many studies have demonstrated that both flavonoids and saponins may possess anti-inflammatory activity (*20*, *29*, *35*), in our experiment these functional components may only play a minor role in anti-inflammation, probably because of the presence of a small amount in isoflavone powder (**Table 2**). Compared to isoflavone (43–48 mg/g) in the powder, lesser amounts of total flavonoids, total phenolic compounds, and saponins were present, which equaled 0.4–3.8, 6.1–15.6, and 0–24.3 mg/g, respectively. Theoretically, a large amount of NO and PGE_2 can be induced in the presence of LPS or cytokine (*11*). Also, PGE_2 can be formed through metabolism of arachidonic acid catalyzed by COX-2. Several authors have demonstrated that both genistein and daidzein could inhibit COX-2 activity (*29*), but the inhibition of NOS activity by isoflavones was seldom conducted. Nevertheless, as mentioned in the preceding section, a study by Raso et al. (*32*) did show a reduction in expression of the iNOS protein by flavonoids. Thus, it may be postulated that the anti-inflammation mechanism of isoflavones may involve inhibition of activity of NOS and COX-2, which in turn reduces production of NO and PGE_2, respectively.

In conclusion, both isoflavone powder produced from soybean cake and genistein standard were effective in decreasing the leukocyte number in mouse blood and lowering the secretions of IL-1β, IL-6, NO, and PGE_2 in peritoneal exudate cell supernatant and peritoneal exudate fluid. The results of this study may be used as a basis for the possible production of functional foods in the future.

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