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Improvement of Menopausal Signs by Isoflavones Derived from *Sophorae fructus* in Ovariectomized Female Rats and the Antioxidant Potentials in BV2 Cells

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The aim of this study was to determine if the isoflavones from *Sophorae fructus* (SISO) have potential clinical benefit in hormone replacement therapy (HRT) for the treatment of menopausal signs, such as the levels of total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL) and follicle stimulating hormone (FSH). An additional aim was to present the potential antioxidant effect of SISO in a microglial cell line. For the animal model, the ovaries were removed from adult rats and the indicators of menopause were measured at the pre- and post-administration time points. Although no statistically significant correlation was found, SISO tended to decrease the TC level ($p=0.15$) and the FSH level ($p=0.36$), but to increase the HDL level ($p=0.303$). SISO (< 5 μg/mL) also exerted antioxidant activity on BV-2 microglial cells by inhibiting lipopolysaccharide-induced nitric oxide. This cytoprotective effect was confirmed by trypan blue staining, which was used to test for cellular damage from H_2O_2. In conclusion, this study highlights the anti-menopausal and antioxidant effect of SISO in an ovariectomized rat model, as well as in microglial cells, and provides new clinical targets for the screening of phytoestrogens as potential candidates for HRT in menopausal women.

Key words: *Sophorae fructus*, Phytoestrogen, Hormone replacement therapy, Menopause, Climacterics

INTRODUCTION

Estrogen, which is produced in the ovaries and testis, has biological effects in the body in addition to its reproductive effects. The level of this sex hormone decreases as a woman ages. This brings about menopause, which naturally occurs after the woman's supply of follicles has been depleted and menstruation ends. In a clinical point of view, menopause is diagnosed after 12 months of amenorrhoea. The average age at menopause is approximately 51 years (Greendale *et al.*, 1999). The clinical symptoms appearing between the pre- and post-menopausal period are regarded as being significant because women may sometimes begin to experience symptoms prior to the actual cessation of their regular menstrual cycles. The climacteric is a general term used for the time from the period of this transition to the early postmenopausal phase of a woman's reproductive life cycle. The typical climacteric symptoms include hot flushes or flushes, insomnia, weight gain and bloating, mood changes, irregular menses, mastodynia, and headache. As an irregular ovarian function and considerable estrogen level fluctuations cause the climacteric symptoms during menopause, stopping the hormone fluctuation with oral contraceptive pills (OCPs) and hormone replacement therapy (HRT) alleviates the climacteric symptoms that help maintain the estrogen level by the continued ingestion of exogenous hormones. HRT with estradiol-17β (E_2-17β) ester or conjugated estrogen can effectively prevent these climacteric complaints, particularly the hot flushes (Greendale *et al.*, 1998).

Regardless of its clinical value in treating menopause, HRT has been implicated in a number of adverse outcomes, including endometrial cancer and an increased risk of breast and ovarian cancer (Beral *et al.*, 1999; Tavani and La Vecchia, 1999). Therefore, there has been a great deal of interest in plant-derived estrogens, known as phytoestrogen, which have an estrogen mimicking effect. This

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focus on phytoestrogen was started with the pioneering work on cancer research associated with hormone deficiency and soy intake (Adlercreutz , 1998). Currently, there are four known families of phenolic compounds, or phytoestrogens, produced by plants, isoflavonoids, stilbens, lignans, and coumestans, which are believed to have an estrogen-mediated response in different ways (Teresa *et al.*, 2004).

Among the alternative therapies in menopause, isoflavones appear to be selective estrogen receptor modulators (SERM) with agonist activity that is modest at the beta estrogen receptor (ERβ) and weak at the alpha estrogen receptor (ERα) (Kuiper *et al.*, 1998). In addition, isoflavones appear to have both antiestrogenic and estrogenic properties (Cassidy *et al.*, 1994), which suppress the estrogen-induced proliferation of human breast cancer cells (Brownson *et al.*, 2002). In phytoestrogens, soy has received considerable attention as a source of phytoestrogen (Fitzpatric, 2003). It was previously shown that *Sophorae fructus* isoflavones (SISO) have an estrogen-like effect in rat bone marrow cells (Joo *et al.*, 2004). Therefore, this study examined the anti-osteoporotic effect of SISO and compared this effect with the effect of estradiol (E2) and soybean-derived isoflavones. The results showed that SISO enhanced the production of IGF-1 and TGF-β while suppressing osteoclastogenesis, which is closely associated with the development of osteoporosis that occurs during the menopausal period. It is possible that SISO has a better anti-climacteric potency in menopausal animal models than that of soybean isoflavones, and might be of clinical interest as an estrogen replacement therapy.

This study focused on establishing the potential activity of SISO in several menopausal markers, such as body weight and the levels of total cholesterol (TC), high density lipoprotein (HDL), and follicle stimulating hormone (FSH). As a secondary endpoint, the anti-oxidant effect of SISO was examined by analyzing the changes in the hydrogen peroxide (H_2O_2) levels. In addition, this study investigated the *in vitro* antioxidant potential of SISO in the brain macrophage cell line (BV-2) for the probable application of SISO in postmenopausal neurodegenerative diseases such as Alzheimer's disease.

MATERIALS AND METHODS

Chemicals and cell culture

SISO (high glucosidic isoflavone with >83% genistein) and soybean isoflavone, BISO, (comparable control, active ingredient level) were obtained from Rexgene Biotech Inc, Korea. The 17β-Estradiol, E2, (>98%) was purchased from Sigma, U.S.A., as white powder. For a stock solution, 17β-Estradiol was primarily dissolved in ethanol (20 μg/mL), and mixed with PEG400 and saline (1:4 ratio), whereas the isoflavones were dissolved in distilled water. The BV-2 cells were grown in 10% FBS α-MEM (Cellgro, U.S.A.) for three days after being thawing and refreshed with 5% FBS α-MEM on day four. All the studies were performed using 1-5% FBS α-MEM media after incubation overnight prior to use because < 5% FBS did not activate the BV-2 cells. For microglial activation, 5 μg/mL lipopolysaccharide (LPS; Sigma, U.S.A.) was added to the cell culture. The nitric oxide (NO) donor was 100 μM of *S*-nitroso-*N*-acetylpenicillamine, SNAP (Sigma, U.S.A.).

Experimental animals

Ten-week old female Sprague-Dawley rats (Orient, Inc., Korean distributor for Charles River, Canada) initially weighing ca. 190-220 g were housed in stainless-steel, wire-bottom, mesh cages under an optimum environment at 24°C and 50% humidity with a 12 h/12 h light dark cycle. Upon arrival, the rats were housed in a stress-free environment for 5 days for acclimatization. Their food intake, stools, and behavior were monitored three times a week and rats with any abnormality were excluded. The rats underwent an ovariectomy after the one-week stabilization period, and were randomly assigned to five groups based on the monitored information: (A) ovariectomized (OVX-cont; n=6), (B) OVX dosed with 500 mg/kg SISO (OVX-SISO; n=6), (C) OVX dosed with 500 mg/kg BISO (OVX-BISO; n=6), (D) OVX dosed with 20 μg/mL E2 (OVX-E2; n=6), and (E) non-OVX dosed with normal diets (non-OVX; n=6). The test substance was administered orally to all the rats except for the OVX-E2 group, in which it was injected subcutaneously. Blood was collected from the jugular vein at 0, 2, and 4 weeks and centrifuged for 5 min. Serum taken from 1 mL of whole blood was used for the measurement.

Surgery

The rat was anesthetized with diethyl ether, the hair of the surgical area was shaved, and the area was painted with 70% ethanol as a skin disinfectant. An abdominal midline incision was made through the skin, and the exposed ovaries were pulled into the incision through the thin muscle wall by retracting the skin laterally toward either side and excised after ligating the upper horn of the uterus. The wounds were closed with a complete suture and sprayed with an antiseptic spray after painting with a tincture of iodine. The rats were then housed for a two-week period with normal feeding and strict monitoring.

Measurements for total cholesterol, HDL, FSH, NO, and RT-PCR

Thirty female SD rats were weighed three times on an electronic balance before surgery and the weight was

monitored once a week for one month. For TC and HDL measurements, a powerful blood test system, Cardio-Check (Polymer Technology System, U.S.A.), consisting of separate test strips was used according to the manufacturer's instructions. Briefly, the serum obtained was applied to the inserted CardioCheck strip, and the results were read on the screen and automatically corrected and measured within two minutes. The test strip was removed and the measurement was repeated three times per rat. The low density lipoprotein (LDL) levels were obtained by subtracting the HDL levels from the TC value for each rat.

FSH was assayed using the kits (FSH ELISA Kit) purchased from Alpha Diagnostic Int'l, U.S.A.. Briefly, blood was collected by a jugular venipuncture, allowed to clot, and the serum was separated by centrifugation at 740 g for 10 min at room temperature. The unused sera were stored at -20°C and used up to the date of analysis (within four weeks). Fifty microliters of the standard, control, and serum samples were pipetted into the microtiter well strips on the plate in duplicate, and 100 μL of the Ab-enzyme conjugate was pipetted into each well with gentle stirring. The plate was covered and incubated for 60 min at room temperature. The wells were then aspirated and washed three to five times with 300 μL of distilled water. The plate was covered and incubated for 30 min at room temperature after applying 200 μL of the TMB substrate solution. The reaction was quenched by adding 50 μL of a stopping solution and the absorbance was measured at 450 nm using an ELISA reader, Spectra Max 340 (Molecular Devices, U.S.A.).

The antioxidant effect was measured by using a colorimetric hydrogen peroxide kit (Assay Designs, USA) according to the manufacturer's instructions. Briefly, the sera were diluted in a 50 mM phosphate, pH 6.0 solution and a hydrogen peroxide standard was prepared at concentrations of 3,400 (100 μM), 1,700 (50 μM), 850 (25 μM), 425 (12.5 μM), 215.5 (6.25 μM), and 106.25 (3.125 μM) ng/mL. Fifty microliters of the sample diluents were pipetted into duplicate wells and 100 μL of a color reagent was pipetted into the blank, standards and sample wells. The side of the plate was tapped for 10 sec for mixing and the mixture was incubated for 30 min at room temperature. The optical density was read at 550 nm.

For the *in vitro* study in BV2 cells, an NO (nitric oxide) scavenging test, an NO^- assay, RT-PCR for iNOS and trypan blue cell staining for cytotoxicity induced by hydrogen peroxide (H_2O_2) were performed. Briefly, the NO scavenging test was conducted using a modification of the method in the Griess Reagent System using 100 μM SNAP as an NO donor. The values were analyzed by comparing the absorbance measured at 540 nm. The NO concentration in the media was measured using the Griess Reagent System and the iNOS expression was measured at the gene level using RT-PCR. RT-PCR was conducted under the most optimistic conditions for the target products of iNOS (382bp), sense, 5'- ggc acc gag att gga gtt cg -3', anti-sense, 5'- gcc aga tgt ggg tct tcc ag -3' and β-actin (186bp), sense 5'- tga ccg agc gtg gct aca gc- 3', anti-sense 5'- acc gct cat tgc cga tag tg-3' (30 cycles with annealing at 58°C for 20 s), as described previously (Joo *et al.*, 2004).

Trypan blue staining

The cells were subcultured into 4-well chamber slides and grown to 90% confluence. The growth media was then removed and an acclimatization media was added with and without H_2O_2 (200 μM) in the presence or absence of 1 to 5 μg/mL SISO. After 4 h incubation, the media was removed and the cells were incubated for 1 min after adding 100 μL of a 0.4 % trypan blue solution along with 300 μL of 1% PBS into each well. The cells were observed under an inverted microscope (Olympus CK30, Japan) at ×100 magnification.

Statistical analysis

The results are presented as the mean ± SEM (standard error of the mean). A P value < 0.05 was considered significant. Analysis of the variance (ANOVA) and paired *t*-test were used to examine the data using the SPSS and GraphPad statistics programs.

RESULTS

Improvement of menopausal indices *in vivo*

Table I shows the means ± standard deviations of the TC, HDL, and LDL levels for the five groups measured at the following three time points: pre-, and post-administration. The different scores in the bar graph between the OVX-control and OVX-SISO group were compared (Fig. 1). The changes in the TC levels in the OVX-SISO ($p=0.340$, pre vs. post) and OVX-BISO ($p=0.463$) groups compared with the OVX-control group showed that the isoflavones from either the soy bean or *Sophorae fructus* may play a certain role in lowering the TC level ($p=0.629$). However, the non-OVX group did not show a change in the total TC (Table I). Moreover, during the administration of the isoflavones, the HDL levels increased with time. In contrast, the reduced TC level was due to the relative decrease in the LDL level. Fig. 1 presents data of the control and OVX-SISO groups that show statistically significant ($p < 0.05$) difference from the OVX-SISO group.

The patterns of FSH level between before and after were similar in all the study groups, except for the OVX-SISO group which showed a decrease in the post-administration (Fig. 2). The trend of the FSH levels found after administering SISO suggested that isoflavone derived

Table I. Impact of the diets on the levels of serum total cholesterol (TC), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) in rats (mean ± S.D.) between the pre- and post-diet condition

Group	Week 0 (TC/HDL/LDL)	Week 4 (TC/HDL/LDL)
OVX-cont	161.0 ± 15.9/ 43.5 ± 4.0/ 117.5 ± 15.1	170.2 ± 32.8/ 42.5 ± 4.7/ 127.7 ± 31.4
OVX-SISO	204.5 ± 9.9/ 34.7 ± 4.3/ 169.8 ± 10.2	179.0 ± 31.8/ 55.2 ± 19.5/ 123.8 ± 14.6
OVX-BSIO	190.0 ± 16.8/ 34.7 ± 5.8/ 155.3 ± 18.0	175.0 ± 31.9/ 54.0 ± 16.2/ 121.0 ± 16.5
OVX-E2	195.0 ± 6.8/ 38.8 ± 6.1/ 156.2 ± 10.9	198.0 ± 31.2/ 63.0 ± 12.9/ 135.0 ± 23.7
non-OVX	198.5 ± 37.3/ 52.7 ± 15.4/ 145.8 ± 24.7	203.3 ± 29.3/ 61.9 ± 9.2/ 141.4 ± 23.0

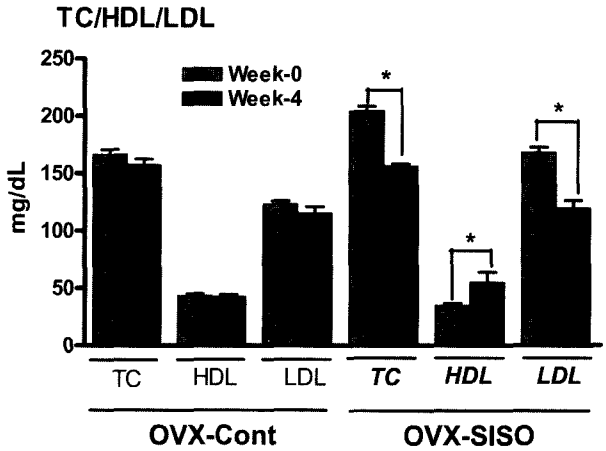

Fig. 1. Changes in the total cholesterol, high density lipoprotein, and low density lipoprotein levels in the SISO group. OVX-cont, ovariectomized; OVX-SISO, OVX dosed with isoflavones from *Sophorae fructus*. OVX was performed on 11 week-old rats after a 1 week stabilization period. The 4-week administration of each sample commenced at the age of 14 weeks. The values shown represent as the mean ± S.D. Statistical significance was evaluated using paired *t*-test; * 0.05.

from *Sophorae fructus* might have greater clinical potential than that derived from the positive control (OVX-BISO) in the anti-menopausal treatment. The body weight of OVX-SISO rats was measured in order to determine whether SISO prevents weight gain after estrogen cessation and whether the weight change has any correlation with TC, HDL and FSH levels. In the SISO group, the weight had decreased by week 4 (p=0.153) suggesting that SISO

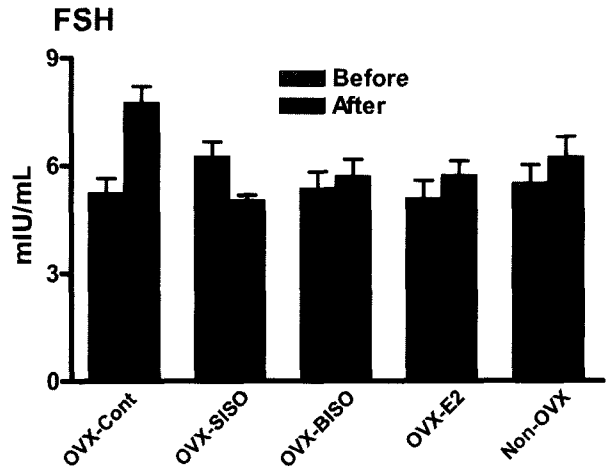

Fig. 2. Effect on the follicle stimulating hormone in all tested groups. The values were measured at week-0 and week-4. The hormonal changes were analyzed after either the cessation or continuance of estrogen release. OVX-cont, ovariectomized; OVX-SISO, OVX dosed with isoflavones from *Sophorae fructus*; OVX-BISO, OVX dosed with isoflavones from soybean; OVX-E2, OVX dosed with estradiol 17β; non-OVX, non-ovariectomized dosed with normal food. The values shown represent the mean ± S.D.

Table II. Correlation between climacteric signs and weight changes

Endpoints	Pre (Mean ± SD)	Post (Mean ± SD)	Probability value (pared t-test)	Trends
FSH (mIU/mL)	6.3 ± 2.5	4.9 ± 1.7	P=0.36	↓
T. Cholesterol (mg/dL)	204 ± 9.9	179 ± 31.8	P=0.15	↓
HDL (mg/dL)	34 ± 4.3	55 ± 19.5	P=0.303	↑
Weight (g)	305 ± 22	287 ± 23	P=0.153	↓

regulates the weight gain, but no correlation between the weight and TC, HDL and FSH levels was found (Table II).

Antioxidant effect of SISO *in vitro*

A murine cell line, BV-2, was used to investigate on the effect of SISO *in vitro*. SISO was added to the cells in the presence or absence of LPS, which is known to induce NO via the expression of inducible NO synthase (iNOS) in the microglia. The effect of NO scavenging by SISO was first investigated by analyzing various SISO concentrations in a Griess Reagent System (Fig. 3). As shown in Fig. 3, SISO effectively scavenged NO at all concentrations ranging from 0.5 to 100 μg/mL, with the most significant scavenging effect being observed at 1 to 5 μg/mL ($p < 0.001$). Therefore, the NO induced from the activated BV-2 was further examined to determine if this induction was regulated by SISO (Fig. 4A, 4B). SISO attenuated the expression of iNOS from BV-2 that was activated by LPS (Fig. 4B). No statistically significant difference between the control and SISO group was found, indicating that

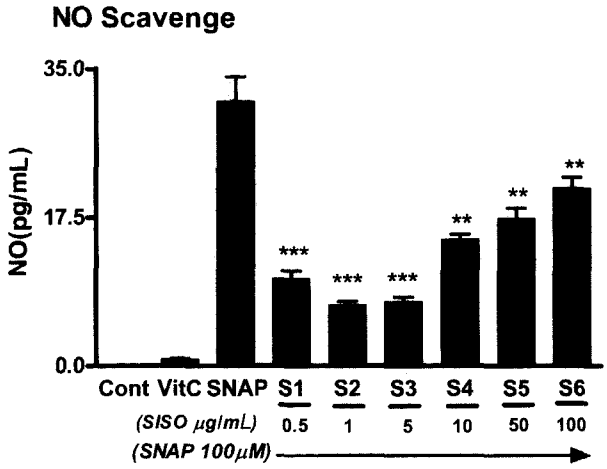

Fig. 3. Detection of the NO scavenging potentials by SISO. The test was designed to detect the range of concentrations and the capability of NO scavenging in the presence of various SISO concentrations. Each well in 96-well plates was prepared in triplicate with SISO at various concentrations ranging from 0.5 μg/mL to 100 μg/mL and SNAP was added as an NO donor. After 2 h incubation, the supernatants were analyzed using a Griess Reagent at 540 nm. Vitamin C was used as the positive control and the measured results were normalized to the values from the SNAP single well. The values shown represent the mean ± S.D.; **, $p < 0.01$; ***, $p < 0.001$, vs. SNAP single well.

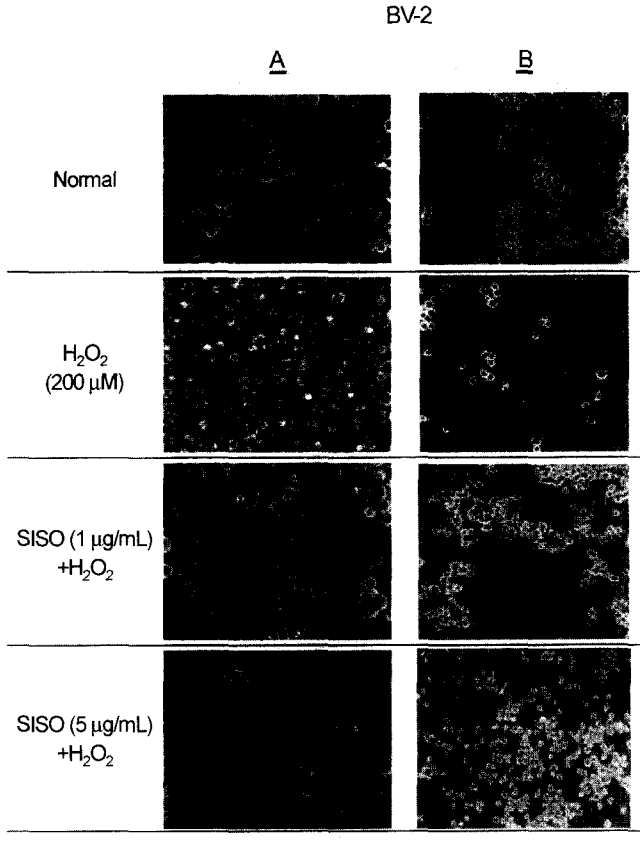

Fig. 5. Cytoprotective effect of SISO. The BV-2 cells were treated with 1-5 μg/mL SISO and a 200 μM H_2O_2 solution was added. The cells were then incubated for 2 h at 37°C in a 5% CO_2 environment. After incubation, the cells were observed under inverted microscopy by comparing the morphological changes in the unstained cells (A) and in the cells stained by 0.4% trypan blue, as described in Materials and Methods (B). The microscopic observations were performed at × 100.

SISO can maintain a gene expression level as low as that found in the control group. In parallel, the level of NO production in the BV-2 cells (Fig. 4A) was measured using a Griess Reagent System. As shown in Fig. 4A, SISO effectively inhibited the NO production at all doses with statistical significance at 5 μg/mL ($p < 0.05$). Finally, the cytoprotective effect of SISO in BV-2 cells was examined using trypan blue staining (Fig. 5). The microscopic

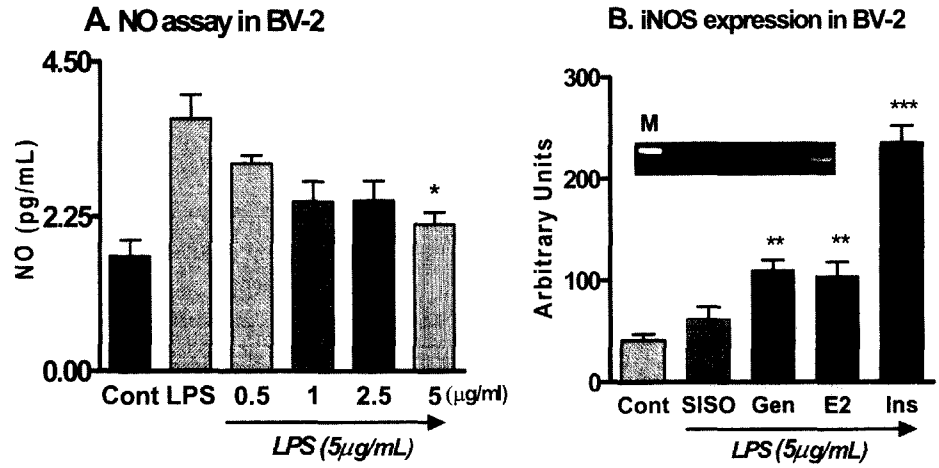

Fig. 4. Lipopolysaccharide-induced NO production and iNOS in BV-2 cell lines. In order to determine the most potent SISO concentration, the cells were treated with 0.5 to 5 μg/mL of SISO (A) and the iNOS expression level was analyzed using RT-PCR. LPS at 5 μg/mL was added as an activator for the BV-2 cells in all groups (B). The cells were incubated for 24 h at 37°C in a 5% CO_2 environment. Gen, genistein (10 μg/mL); E2, estradiol 17β (0.5 μg/mL). The values shown represent the mean ± S.D.; **, $p < 0.01$; ***, $p < 0.001$ vs. Control.

images of the unstained cells (Fig. 5A) were compared with those stained with trypan blue (Fig. 5B). The images confirmed that SISO could dose-dependently protect the cells from the damage caused by 200 μM H_2O_2.

DISCUSSION

Phytoestrogens are plant-derived compounds that are structurally or functionally similar to mammalian estrogens. Therefore, they are believed to play a clinically important role in preventing menopausal symptoms and osteoporosis (Kronenberg and Fugh-Berman, 2002). More importantly, because long-term HRT treatment for the relief of menopausal symptoms has been associated with an increased risk of breast cancer (Chlebowski *et al.*, 2003), alternative and complementary therapies in menopausal women need to be considered (Kang *et al.*, 2002). Recently, soy, as a source of phytoestrogens, has received considerable attention (Fitzpatric, 2003). Isoflavones, which are SERMs are the most well known phytoestrogen. The aglycones (daidzein and genistein) and glycosides (daidzin and genistin) are the naturally occurring isoflavones that have shown estrogenic activity. Aglycone is the form that is most absorbed into the intestinal mucosa. When ingested in the form of genistin (the glycosidic form), the intestinal bacteria remove the glycosidic moiety and convert it to genistein, which can then be absorbed (Steer *et al.*, 2003).

This study aimed to find evidence supporting the clinical benefit of SISO, which is a group of high glycosidic isoflavones from *Sophorae fructus*, in relieving the climacteric symptoms of menopausal women, such as flushes which are caused by the changes in the FSH level before or after the cessation of estrogen excretion. First, changes in the TC, HDL, and FSH levels detected in the 4-week period after the administration of SISO were examined to determine any correlation between these level and the weight changes (Table II). However, no statistically significant correlation was found ($p > 0.05$). The trends of these menopausal indices showed that the levels were compensated for at the time of SISO administration. Interestingly, the TC level was downregulated ($p=0.15$), whereas the HDL level was increased ($p=0.303$). This suggests that the relative LDL level decreased. Moreover, the FSH level, which is associated with flushes during the menopausal period, was also downregulated. This indicates that SISO plays a role as a substitute for estrogen in OVX rats.

To summarize, SISO appears preferable to other alternative and complementary therapies, including soybean isoflavones. Moreover, SISO may have potential effects on both climacteric and menopausal symptoms when compared with positive controls such as soybean isoflavones and E2. This *in vitro* study showed that SISO can scavenge NO in a modified NO scavenging system, as confirmed by the gene expression level (iNOS expression) and the detection of NO produced from the BV-2 cells (Figs. 3, 4). The beneficial effects of SISO on cytoprotection were also examined by trypan blue staining in the presence of H_2O_2. Fig. 5 shows that 5 μg/mL SISO gave maximum cell protection against a high H_2O_2 concentration in a dose dependant manner (Fig. 5). These results support the hypothesis that isoflavones, particularly SISO, can effectively act as an 'estrogen-like' compound. Moreover, SISO has greater potential than soybean isoflavones in the treatment of menopausal symptoms. In conclusion, SISO may ameliorate the menopausal signs, including the climacteric symptoms, and elevate the antioxidative defense in microglial cells against oxidative stress which may prevent neuron cells from the increased oxidants in the brain after menopause. However, various studies with more precise results are required before these isoflavones can be applied to HRT therapy because the mechanisms and potencies of phytoestrogens have not been completely clarified and they may have potential endocrine disrupting functions (Wuttke *et al.*, 2003).

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