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Effects of Dietary Soy Protein and Soy Isoflavones on Cerebral Infarction Size and Antioxidant Enzyme Activities in a Rat Focal Ischemia Model*

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In this study we investigated the neuroprotective, antioxidative, and hypocholestrolemic effects of dietary soy protein and soy isoflavone in a rat focal brain ischemia model. Weaning Sprague-Dawley rats were fed a 20% casein-based diet (CA), 20% soy protein-based diet (SP), or 0.2% soy isoflavones-supplemented diet (ISO) for 6 weeks. The cortical infarction volume of the ISO group was significantly lower than that of the SP group. The thiobarbituric acid reactive substances (TBARS) were considerably lower in the ISO group than the CA group. Glutathione peroxidase activities of the SP group were notably higher than those of the CA group. Acetylcholinesterase (AchE) activities of the SP group were significantly decreased compared to the CA group. LDL cholesterol levels and LDL/HDL ratios of the ISO group were lower than those of the CA and SP groups. Our results collectively suggest that soy isoflavones may contribute to neuroprotection by reducing the TBARS and serum LDL/HDL ratio, whereas soy protein may be associated with the regulation of cognitive functions by modulating AchE activity.

Key words: Acetylcholinesterase, Infarction volume, LDL-cholesterol, Soy isoflavones, Soy protein

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INTRODUCTION

Stroke is a leading cause of death worldwide and causes serious disabilities in survivors. The incidence of stroke is associated with numerous factors, such as oxidative stress, dietary components, and lifestyle factors.[1,2] A number of dietary protective factors against the development of stroke have also been reported.[3-7]

Soybeans are nutrient-dense, and a unique source of isoflavones. Beneficial effects of soybean on mortality rates in Japan were reported.[8] People in Western societies have also recognized the benefits of soy protein.[9] Studies have found that the replacement of an animal protein-based diet with a soy protein diet reduced hyperlipoproteinemia and atherosclerosis.[10]

It has been proposed that the beneficial effects of soy and soy isoflavones (genistein and diadzein) are due to their antioxidant properties; this suggestion is most convincing in light of the apparent reduction of peroxide levels by these compounds.[11] Soy isoflavones also enhance antioxidant enzyme activity,[12,13] particularly that of glutathione peroxidase (GPx).[14]

Recently, there is growing interest in the cognitive function and beneficial effects of soy isoflavones on neurodegenerative disease such as dementia and stroke. A number of studies have reported that dietary soy-derived phytoestrogens (mostly isoflavones) influence learning, memory and cognitive functions.[15-17] More recent studies have shown that soy isoflavones protect neural cells from apoptosis[19,20] and oxidative stress,[21] enhance choline acetyltransferase activity and inhibit acetylcholinesterase (AchE) in rat cortex[22] and increase the basal nitric oxide activity in the basilar artery of male rats.[23]

Soy protein and soy isoflavones have been studied for their potential beneficial effects against cancers,[24] hypercholesterolemia[25-30] and age-related disease,[31,32] however, little is known about the influence of soy isoflavones

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on neuroprotection, antioxidant, and dyslipemic properties in focal ischemia models. Moreover, no conclusive data have been reported on the anti-cholinesterase effects of soy protein and soy isoflavones in stroke, although loss of cholinergic innervation by reduced choline acetyltransferase and elevated AchE activity, is correlated with the degree of dementia[33] and the central cholinergic system is considered to be the most important neurotransmitter involved in the regulation of cognitive function.[34]

Accordingly, this study was performed to investigate the neuroprotective effects of dietary soy protein and isoflavones in a rat focal brain ischemia model. The model used in our study was based on a model by Nagasawa and Kogure.[35] The model is designed for studying experimental cerebral ischemia in order to simulate cerebrovascular occlusive disease in humans.[36] It has previously been used to assess the neuroprotectve effects of therapeutic treatments[37,38] and diets.[39-41] We additionally analyzed the mechanisms by which dietary soy protein and soy isoflavones affect antioxidant activity and the hypocholesterolemic and cognitive function in this model.

MATERIALS AND METHODS

1. Animals and Diet

Experiments were performed in accordance with procedures outlined in the Guidelines for Animal Experimentation (Seoul National University). Male Sprague-Dawley rats at the point of weaning (4 weeks of age, Daehan Biolink, Chungbuk, South Korea) were divided into three dietary groups and were fed these diets for the next 6 weeks. Rats consumed food and water *ad libitum*. One group was fed a regular diet based on purified rodent diet with casein protein (CA) (AIN-93 G, Dyets, PA, USA) as the control protein, with some modifications (Table 1). The second group was fed a soy protein diet, which was substituted for casein protein at a level of 20% (SP) (Table 1). The third group was supplied soy isoflavones (Dr. Chung's Food Company Limited, Choongbuk, South Korea) added to the CA diet at a level of 0.2% (ISO) (Table 1). The semipurified isoflavone extract added to the casein protein was made from an alcohol extract from a soygerm. The percentage of distribution of genistein, daidzein, and glycitein within the semipurified isoflavone extract was 4.5, 16.0, and 9.5%, respectively. The isoflavone extract was present in the aglycone form.

Table 1. Composition of the experimental diet

(g/100g)

Ingredient	CA	SP	ISO
Casein	20.0		20.0
Soy protein	-	20.0	-
Corn starch	62.7	62.7	62.7
Cellulose	5.0	5.0	5.0
Soybean Oil	7.0	7.0	7.0
Vitamin Mixture	1.0	1.0	1.0
Salt Mixture	4.0	4.0	4.0
DL-methionine	0.3	0.3	0.3
Soy Isoflavones	-	-	0.2

CA, 20% casein-based diet; SP, 20% soy protein-based diet; ISO, 0.2% soy isoflavones-supplemented diet

2. Focal Brain Ischemia Model

Focal brain ischemia was induced using a model of the middle cerebral artery (MCA) occlusion.[35,40] In brief, the rats were anesthetized with halothane (2% in a mixture of 70% N_2O and 30% O_2). Body temperature was maintained with a heating pad at 36.5~37.5 ℃. After median incision of the neck skin, the right carotid artery was exposed, leaving the vagus nerve intact. A silicone rubber cylinder attached to a nylon surgical thread was introduced above the bifurcation of the internal carotid artery in order to block blood flow to MCA from the collateral circulation from the left side of the brain. The cylinder was made of 4-0 nylon surgical thread 16 mm long coated with silicone (Xantopren, Heraeus Kulzer, South Bend, IN, USA), and mixed with hardener (Elastomer Activator, Heraeus Kulzer) in order to thicken the distal 5 mm section.[35] After the insertion of the cylinder, the internal carotid artery was ligated distal to the point of insertion. Surgery was performed within 10~15 min, with no bleeding. Following surgery, anesthesia was discontinued.

All rats exhibited neurologic deficits characterized by left hemiparesis with upper- extremity dominance. Circulation was restored by pulling the thread out of the internal carotid artery after 2 hours of MCA occlusion. Rats were given food and water until the next procedure.

3. Infarction Size

At 24 hours after reperfusion, animals were decapitated and brains were removed. After brief incubation in ice saline, seven 2 mm-thick coronal brain slices were made for each animal (n=8, CA group; n=6, SP group; n=5, ISO group). Brain sections were photographed using a CCD camera after staining with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Chemical, Saint Louis, MO, USA) for 60 minutes at room temperature.[42-44] The

infarcted area was measured using an image analysis system (BMI plus 1.2, Bummi Universe, Korea), and the volume was calculated. The cortex area was measured separately afterwards.

4. Sample Preparation

For biochemical assay, the frontoparietal cortex, somatosensory area and lateral segment of caudate putamen were separated. These areas were selected in view of previous reports that infarction areas were localized in these regions.[35] We did not include the medial segment of the caudate putamen, since infarction in this area was observed rarely and only in severely damaged rats.[35,45] We separated only the right cerebral cortex (corresponding to the infarction side) and placed the specimens on ice (n=6, CA group; n=7, SP group; n=6, ISO group).[35] Tissues were weighed and homogenized in ice-cold 0.05 M Tris-HCl buffer (pH 7.5). Homogenates were adjusted to a final concentration of 100 mg tissue/ml of buffer and were then used for the thiobarbituric-acid-reactive substances (TBARS) assay. Then, brain homogenates were fractionated by differential centrifugation, as described previously by Roy *et al.*[46] The homogenates were centrifuged at 1000×*g* for 10 min at 4 ℃, and the supernatant was used to assess the catalase (CAT) and superoxide dismutase (SOD) activity. The remaining supernatant was further centrifuged at 12500×g for 30 min at 4 ℃. The resultant supernatant containing microsomes and cytosol was employed for the GPx assay.

5. Biochemical Analysis

1) TBARS

TBARS were measured in the brain homogenates using slight modifications to the method of Buege and Aust.[47] Briefly, aliquots containing 0.1~0.2 mg protein were mixed with 150 mM KCl, 0.05 M Tris-HCl buffer (pH 7.5), and 15% (w/v) trichloroacetic acid (TCA)-0.375% (w/v) thiobarbituric acid (TBA)-0.25 N HCl (Sigma Chemical, St Louis, MO, USA). The mixture was kept in a boiling water bath for 15 min to obtain the red color of various compounds that reacted with TBA. After centrifugation, the color was measured at 535 nm. The amount of TBARS was calculated using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

2) SOD Activity

In keeping with the procedure of Misura and Fridovich,[48] SOD activity was measured at 30 ℃ by determining the inhibition of epinephrine autoxidation in 50 mM sodium carbonate buffer (pH 10.2) and approximately 30 μl of 10 mM epinephrine (Sigma Chemical, Saint Louis, MO, USA), which gave an initial autoxdation of 0.025 absorbance units per minute at 480 nm. The amount of SOD required to inhibit the rate of epinephrine autoxidation by 50% was defined as one unit of activity and was expressed as units per milligram of protein.

3) CAT Activity

Following the method of Abei,[49] the reaction was initiated by adding H_2O_2 in 50 mM phosphate buffer (pH 7.0) in the presence of tissue extracts. The degradation of H_2O_2 was monitored at 240 nm and 20 ℃, and an extinction coefficient of 43.6 $M^{-1}cm^{-1}$ was used to calculate units of activity. Enzyme activity was expressed as units per milligram of protein (1 unit =1 μmol of H_2O_2 degraded for 1 min).

4) GPx Activity

GPx activity was measured using the method of Tapple.[50] Activity was assayed using cumene hydroperoxide (Sigma Chemical) as a substrate. NADPH oxidation was monitored at 340 nm and 37 ℃, and an extinction coefficient of 6.22 $M^{-1}cm^{-1}$ was employed to calculate units of activity. Enzymatic activity was expressed as nanomoles of NADPH oxidized per minute.

5) AchE Activity

The hydrolyzing activity of AchE (the classical enzyme in the cholinergic neurohormonal system) that inhibits the prolonged action of the cholinergic neurotransmitter, acetylcholine, was measured in whole brain.[51] Whole brain was selected, since AchE activity is high in the cerebral cortex, corpus striatum and hippocampus regions.[52,53]

After rats were sacrificed, ischemic whole brains were mixed in 0.1 M phosphate buffer (pH 8.0) and homogenized (n=5, CA group; n=6, SP group; n=3, ISO group). AChE was assayed in a spectrophotometer at 412 nm, according to the method of Ellman *et al.*[54] with acetylthiocholine iodide (Sigma Chemical) as a substrate in dithiobisnitrobenzoic acid (DTNB) (Sigma Chemical). The extinction coefficient of 5-thio-2-nitro-benzoic acid produced from the substrate reacting with DTNB that was used to calculate units of activity was 1.36×10^{-4} $M^{-1}cm^{-1}$.

6) Protein Assay

Protein amounts of brain fractions were determined using the method of Lowry *et al.*[55]

7) Plasma Lipid Concentrations

After rats were sacrificed, blood was collected by cardiac puncture (n=14, CA group; n=13, SP group; n=14, ISO group). Total cholesterol, low-density lipoprotein, and high-density lipoprotein in plasma were measured using lipoprotein assay kits (Waco, Osaka, Japan).

6. Statistical Analysis

All results were expressed as means and standard deviations. The one-way ANOVA test was used for statistical analysis. Duncan's multiple-range test was employed to determine statistical significance at $p<0.05$. Statistical analyses were performed by SAS version 8.2 for Windows software.

RESULTS

1. Infarction Size

The cerebral infarction volumes of the cerebral cortex on the right side were significantly smaller in the ISO group than in the SP group ($p<0.05$). However, there were no significant differences between the SP and CA groups or between the CA and ISO groups (Figure 1).

2. TBARS

The TBARS level was clearly lower in the ISO group than in the CA group ($p<0.05$). Although there were no significant differences in TBARS between the CA and SP groups, the levels were lower in the SP group (Figure 2).

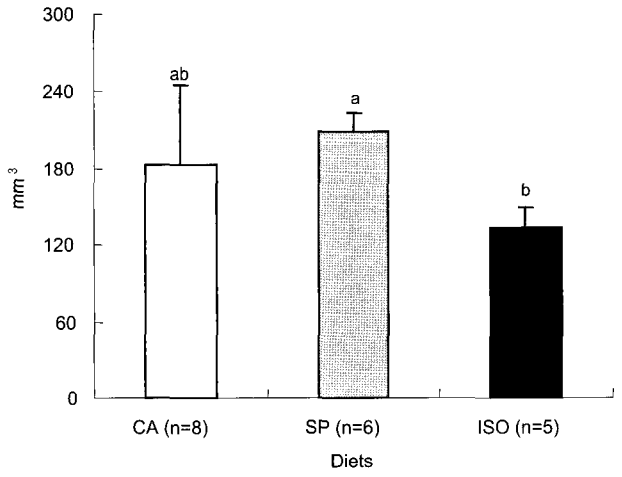

Fig. 1. Cortex infarction volumes in the CA, SP and ISO groups
Bars show means ± SDs in the CA, SP and ISO groups.
Means labeled with different letters are significantly different ($p<0.05$) by Duncan's multiple range test, while those with the same letter are not.
CA, 20% casein-based diet; SP, 20% soy protein-based diet; ISO, 0.2% soy isoflavones-supplemented diet

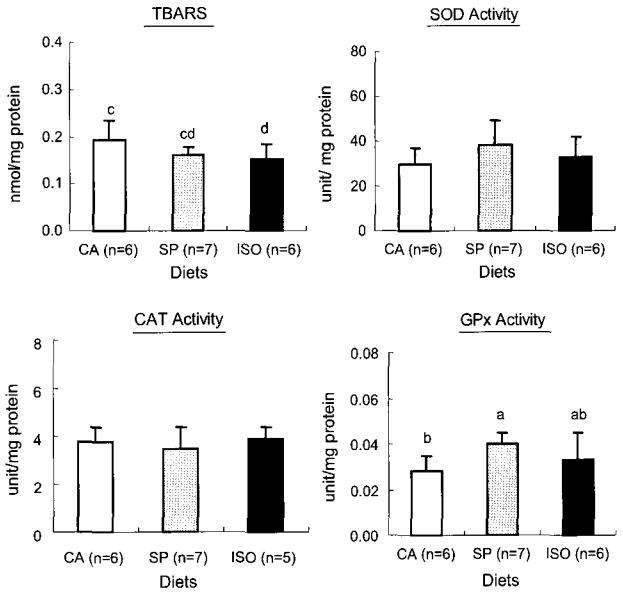

Fig. 2. TBARS levels and SOD, CAT, and GPx activities in the CA, SP, and ISO groups
Bars show means ± SDs in the CA, SP and ISO groups.
Means labeled with different letters are significantly different ($p<0.05$) by Duncan's multiple range test, while those with the same letter are not.
CA, 20% casein-based diet; SP, 20% soy protein-based diet; ISO, 0.2% soy isoflavones-supplemented diet; TBARS, thiobarbituric-acid-reactive substances; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase

There were no notable differences between the TBARS value in the right cortex and the respective left cortex of each group (data not shown).

3. Antioxidant enzyme activities

GPx activities in the SP were higher than those in the CA group. However, there were no significant differences in SOD and CAT activities among the groups (Figure 2). Activities of antioxidant enzymes between the right cortex and the respective left cortex of each group were relatively similar (data not shown).

4. AchE activity

AchE activities of the SP group were markedly lower than those of the CA group ($p<0.05$), whereas no significant differences were observed between the CA and ISO groups (Figure 3). AchE activity in the right brain did not vary from that in the left brain (data not shown).

5. Lipid compositions

In the ISO group, plasma LDL cholesterol was significantly lower than those of the CA and SP groups ($p<0.05$); although there were no evident differences in plasma total and HDL cholesterol among the groups. The

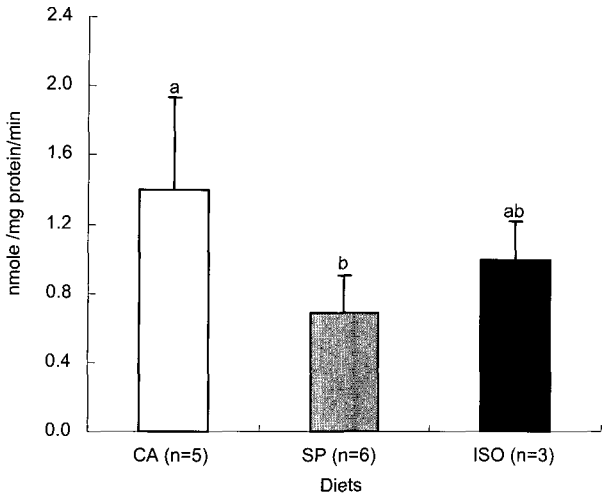

Fig. 3. Acetylcholinesterase activity in the whole brain in the CA, SP and ISO groups

Bars show means ± SDs in the CA, SP and ISO groups. Means labeled with different letters are significantly different ($p<0.05$) by Duncan's multiple range test, while those with the same letter are not. CA, 20% casein-based diet; SP, 20% soy protein-based diet; ISO, 0.2% soy isoflavones-supplemented diet

Table 2. Plasma lipid concentrations in the CA, SP, and ISO groups

	CA (n=14)	SP (n=13)	ISO (n=14)
TC(mmol/l)	76.14 ± 11.35	77.08 ± 13.74	71.57 ± 11.8
HDL-C(mmol/l)	29.86 ± 5.32	29.23 ± 6.22	28.36 ± 4.38
LDL-C(mmol/l)	10.29 ± 2.5[a]	9.46 ± 2.5[a]	6.86 ± 2.93[b]
LDL-C/HDL-C	0.35 ± 0.12[a]	0.33 ± 0.08[a]	0.25 ± 0.11[b]
TC/HDL-C	2.58 ± 0.33	2.68 ± 0.33	2.54 ± 0.3

Data are means ± SDs in the CA, SP, and ISO groups.
Means with different superscript letters are significantly different ($p<0.05$) by Duncan's multiple range test, while those with the same letter are not.
TC, total cholesterol; CA, 20% casein-based diet; SP, soy protein-based diet; ISO, 0.2% soy isoflavones-supplemented diet.

plasma LDL/HDL ratio, an index of cardiovascular disease prevalence, was significantly lower in the ISO group than in the CA and SP groups ($p<0.05$) (Table 2).

DISCUSSION

The present study indicates that dietary soy isoflavone supplementation reduced the cortical infarction volume and suppressed the TBARS and plasma LDL cholesterol levels in a rat brain focal ischemia model. Contrary to expectations, the soy isoflavones had no clear impact on AchE activity, but dietary soy protein decreased AchE activity in the brain.

The infarction volume of the cerebral cortex had a tendency to be smaller in the ISO group than in the CA group. The decreased infarction volume of the cerebral cortex in the ISO group may be partly due to the antioxidant effect of soy isoflavones. TBARS in the ISO group was significantly lower than that of the CA group. Our results are consistent with recent findings showing that soy isoflavones decrease the levels of free radicals in plasma, liver, and brain of rabbits.[56,57]

Soy isoflavones enhance the activities of antioxidant enzymes.[12] In our study, GPx activities in the SP and ISO groups were higher than those of the CA group, suggesting possible enzyme induction in these groups. Our data are in agreement with a previous report in which soy protein increased hepatic GPx activity in rat liver[58] and genistein elevated GPx activities and gene levels in human prostate cells.[41] Currently, there is no clear explanation for why a protein diet only increased GPx activity. GPx activity is possibly associated with the amino acid composition of dietary soy proteins.[59]

Consistent with previous results,[18] our data disclosed that AchE activity of the SP group was significantly lower than that of the CA group. This finding suggests that an increase in acetylcholine in the brain in the SP group may have a favorable effect on cognitive function. On the other hand, our study did not show an association between soy isoflavones and reduced AchE activity. Currently, little is known about the association between cognitive function and soy isoflavones. A previous study reported that soy isoflavones (2 mg/kg BW) decreased AchE activity in liver and brain of rabbits.[57] Recently, Lee *et al.*[22] reported that low-concentration (0.03%) and high-concentration (0.12%) of soy isoflavones inhibited AchE activity in the cortex and hippocampus of elderly SD male rats. However, more recent study reported that low (0.015%) soy isoflavones intake did not have any effect on AchE activity, but, medium (0.075%) and high (0.15%) isoflavones intake enhanced AchE activity in SD male rats without adverse (toxic) effects.[59] There is no clear explanation for why the effects of soy isoflavones on AchE activity in our study were inconsistent with the results in recent reports. It is likely that AchE activity may dependent upon the dosage of dietary soy isoflavones, physiologic condition, and age.

In contrast to soy protein, the soy isoflavones in our study significantly reduced serum LDL cholesterol concentrations and plasma LDL/HDL ratios (an index of cardiovascular disease). Previous data on the hypocholesterolemic effects of soy protein and soy isoflavones on the lipid profile are inconsistent. Some studies have shown that soy protein and soy isoflavones decrease the serum total cholesterol and LDL cholesterol concentrations,[25-28,30,61] whereas others have reported no effect.[62,63]

This discrepancy may be due, in part, to differences among species, sex, periods, and experimental designs. Although the roles of dyslipemia in stroke prevention are much less conclusive than those in heart disease, improvement of serum lipoprotein profiles decreases the risk of the cerebral vascular event in clinical trials.[29] Moreover, higher total cholesterol levels were associated with increased risk of ischemic stroke[64,65] and high HDL-cholesterol levels were related to reduced risk of ischemic stroke.[66] Therefore, the decreased LDL cholesterol and LDL/HDL ratio by soy isoflavones in young adult rats may be partly attributed to stroke prevention in later years.

Although the small sample size and the use of the young adult model in this study necessitates that our findings might be viewed with some caution, our study demonstrates that dietary soy isoflavones tended to decrease the cerebral infarction volume in the rat brain. It appears that changes in both TBARS in the brain and serum LDL cholesterol are at least partly attributable to the decrease in infarction volume in the ISO group. In addition, soy protein decreased AchE activity and this suggests that soy protein may regulate cognitive functions, since AchE activity is the most accepted and recognized therapeutic marker for the development of cognitive enhancers.[34]

The effectiveness of soy isoflavone intake in the prevention of brain ischemia in humans is not yet confirmed, but our results suggest that dietary soy isoflavones exert neuroprotective effects in the rat. However, it is unclear whether several other mechanisms may be involved in the inhibition of the cerebral infarction by dietary soy isoflavones. Other studies should seek to replicate our findings and further explore additional parameters likely to differ between casein and soy isoflavones or soy protein.

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