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Protective effects of a synthetic soybean isoflavone against oxidative damage in chick skeletal muscle cells

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Abstract

The antioxidant and protective properties of a synthetic soybean isoflavone (SI) were investigated using chick skeletal (leg) muscle cells (SMC) isolated from 20-day-old Linnan yellow broiler chick embryo. Skeletal muscle cells were cultured in Dulbecco's modified Eagle's medium treated with 0, 12.5, 25, 50, 75 and 100 μM SI, respectively, under 80 μM $H_2O_2/FeSO_4$ conditions. After 24 h of incubation, SI reduced the loss of SMC under oxidative stress by $H_2O_2/FeSO_4$. The addition of SI significantly promoted SMC proliferation ($P < 0.01$). Upon treatment with 25, 50, 75 and 100 μM SI, the activity of total superoxide dismutase in the supernatant of the media was enhanced by 17.0%, 13.0%, 13.3% and 11.9%, respectively ($P < 0.05$). Compared to the control, the activity of glutathione peroxidase was significantly increased only at 25 μM concentration of SI ($P < 0.05$), and the increment was 90.7%. The activity of catalase was increased by 49.2% and 49.1%, respectively, at 75 and 100 μM SI ($P < 0.01$). The concentration of creatine kinase in the media was decreased by 61.6% and 60.6%, respectively, at 75 and 100 μM SI ($P < 0.01$). The addition of SI did not affect the activity of lactate dehydrogenase in the media. In conclusion, the SI protected skeletal muscle cells from oxidative damage, attributed to its antioxidant activity.
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Keywords: SOD; GSH-px; CAT; CK; T-AOC; LDH; Cell proliferation; Antioxidation

1. Introduction

Great interest has been aroused in antioxidant activity of soybean isoflavones (Doerge & Chang, 2002; Foti et al., 2005; Lee et al., 2005; Win, Cao, Peng, Trush, & Li, 2002; Xu, Ikeda, & Yamori, 2004; Yousef, Kamel, Esmail, & Baghdadi, 2004). Isoflavones have multiple hydroxyl group(s) and act as effective antioxidants by donating a hydrogen atom(s) from their phenolic hydroxyl group(s) to peroxyradicals (Tikkanen, Wahala, Ojala, Vihma, & Adlercreutz, 1998). Record, Dreosti, and McInerey (1995) suggested that genistein, one of the two major components of isoflavone, is an effective scavenger of hydrogen peroxide *in vitro*. It has been demonstrated that isoflavones trap-free radicals (Patel, Boersma, Crawford, Hogg, & Kirk, 2001) and prevent lipid peroxidation in liposomes (Arora, Nair, & Strasburg, 1998a, 1998b) and Caco-2 intestinal cells (Peng & Kuo, 2003). However, very few studies have looked at their antioxidative effects in domestic animal cells.

In defence against oxidative stress, the antioxidant enzyme (AOE) system of cells plays an important role. The antioxidant enzymes include the superoxide dismutases (SODs), catalase (CAT) and glutathione peroxidases (GSH-px) (Aebi, 1984; McCord, 1979; Ursini et al., 1995). CAT and GSH-px convert H_2O_2 to H_2O and the SODs catalyze the dismutation of the superoxide radical anion. Röhrdanz, Ohler, Tran-Thi, and Kahl (2002)

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suggested that SI affect AOE mRNA expression levels in daidzein-treated (300 μM) rat hepatoma H4IIE cells. Choi, Cho, Park, Cho, and Song (2003) observed significant increases in SOD and CAT in genistein-treated (50–100 μM) RAW 264.7 macrophages. Diets containing isoflavone (150 and 250 ppm) showed obvious elevated antioxidant enzymatic levels in various organs of rats that were fed with diets containing partially oxidized oil (Liu, Chang Sam, & Wiesenborn, 2005). Cai and Wei (1996) suggested that dietary genistein enhances the activities of antioxidant enzymes in various organs in SENCAR mice. Most antioxidation studies are conducted in either whole animals or in mice cells or human cells. Little is known about the influence of SI on the AOE system in chick skeletal muscle cells *in vitro*. This is the first study that has evaluated the protective properties of a synthetic soybean isoflavone in chick skeletal muscle cells subjected, *ex vivo*, to oxidative stress. Hopefully, our study will contribute to the application of isoflavones in the meat food industry.

2. Materials and methods

2.1. Chemicals

The soybean isoflavone (SI) was synthesized from resorcin and phenylacetic acid, and its chemical identity was assessed by nuclear magnetic resonance (pending a patent) and the purity was >98%. Dimethylsulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture and treatment

Skeletal muscle cells were prepared from freshly dissected leg muscle of 20-day-old chick embryo, as described by Matsuda, Spector, and Strohman (1983). The muscles from both legs were carefully collected. Periadventitial fat and connective tissue were carefully removed. The muscle was spliced to flat pieces about 4 mm^2, and incubated in a solution of 0.2% (w/v) type I collagenase (Sigma–Aldrich, St. Louis, MO, USA) in DMEM at 37 °C for 2 h on a shaker. Skeletal muscle cells were cultivated in Dulbecco's modified Eagle's medium (DMEM/F_{12}) (GIBCO, Invitrogen Corporation, Shanghai, PR China) containing 10% (v/v) fetal bovine serum and 1% penicillin-streptomycin in 30 mm × 60 mm culture glass dishes. The media were then maintained in a humidified incubator at 37 °C and 5% CO_2 for 48 h. The cells were then transferred using 0.05% trypsin to serum-free DMEM. One hundred microliter of the cell suspension, with an initial concentration of 5×10^5 cells/ml, were inoculated in a well of 96-well microtiter plates for both morphology observation and cell proliferation assay. There were eight replicate wells for each treatment. For biochemical determinations, 3 ml of the cell suspension were added to each well of 6-well microtiter plates. There were six replicate wells per treatment. The SI was diluted in DMSO and added into the serum-free media to reach final concentrations of 12.5, 25, 50, 75 and 100 μM. The control cells were similarly treated with the same amount of DMSO (0.025% (v/v)). Oxidative damage was introduced by adding 80 μM of ferrous ions (as $FeSO_4 \cdot 7H_2O$) and H_2O_2 to each well.

2.3. Observation of cell morphology

The cells were incubated for 48 h in the presence or absence of SI (0, 12.5, 25, 50, 75 and 100 μM). The morphological changes of the cells, for both the control and the treatments, were observed by phase-contrast microscopy (100×) (Axiovert 25 HBO 50/AC, Carl Zeiss, Jena, Gemany).

2.4. Cell proliferation assay

Cell proliferation was determined by the MTT method (Mosmann, 1983) after 24 h of incubation with SI at concentrations of 0, 12.5, 25, 50, 75 and 100 μM. MTT was dissolved in phosphate-buffered saline at 5 mg/ml. Briefly, 10 μl of MTT solution were added to each well, followed by 4 h of incubation. After incubation, MTT-containing medium was discarded, and 100 μl of 10% sodium dodecylsulfate were added to each well to dissolve formazan crystals for 2 h at 37 °C. The concentration of formazan was determined at 490 nm with a microplate reader (Model 550, Bio-Rad, Hercules, CA, USA).

2.5. Biochemical determinations

The supernatant of the cell culture medium was taken for analysis of activities of total SOD (T-SOD), GSH-px, CAT, and the total antioxidant ability (T-AOC), using colorimetric methods with a spectrophotometer (Biomate 5, Thermo Electron Corporation, Rochester, NY, USA). The assays were conducted using the assay kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, PR China) and the appropriate procedures. The activities of creatine kinase (CK) and lactate dehydrogenase (LDH) in the cell medium were determined in a Beckman spectrophotometer (Model CX5, Beckman Instruments, Fullerton, CA, USA) at 340 nm using the assay kits from Beckman Coulter Inc. (Fullerton, CA, USA).

2.6. Statistical analysis

Data were expressed as means ± SE. The effects of SI at the various concentrations were compared with that of control by one-way analysis of variance using computing software SAS (v6.12, SAS Institute, USA). Statistical significance was set at $P < 0.05$.

3. Results

3.1. SMC proliferation under oxidative condition by H_2O_2/$FeSO_4$

Under oxidative damage by H_2O_2/$FeSO_4$, the cells in the presence of SI were lost less compared with that of control, and the cells cultured with 25 μM or 50 μM SI grew obviously better than did the cells treated with other doses of SI or the control (Fig. 1). SMC proliferation was significantly promoted by SI supplementation ($P < 0.01$) (Table 1).

3.2. Biochemical analyses

Activities of the oxidative enzymes, T-AOC, T-SOD, GSH-px and CAT, in supernatant of the media from SMC culture are presented in Table 1. The activity of T-SOD was significantly enhanced by adding SI at concentrations of 25, 50, 75 and 100 μM, and the increments were 17.0%, 13.0%, 13.3% and 11.9%, respectively ($P < 0.05$). GSH-px activity was enhanced only at 25 μM SI ($P < 0.05$, by 90.7%). The activity of CAT was increased by 49.2% and 49.1% at 75 and 100 μM SI ($P < 0.01$), respectively. All doses of SI had no significant effect on the activity of T-AOC ($P > 0.05$).

Addition of SI at 75 or 100 μM significantly reduced CK activity in the media ($P < 0.05$), and the decline was 61.6% and 60.6%, respectively, but not at the other concentrations, compared with the control. Addition of SI at the various concentrations had no significant effect on LDH activity ($P > 0.05$).

4. Discussion

Reactive oxygen species can initiate lipid peroxidation and DNA damage, leading to mutagenesis, carcinogenesis and cell apoptosis, if the antioxidant system is impaired (Devi et al., 2000). Once formed, free radicals attack cell structures, causing damage to cell membrane and enzyme systems (Giray, Gurbay, & Hincal, 2001).

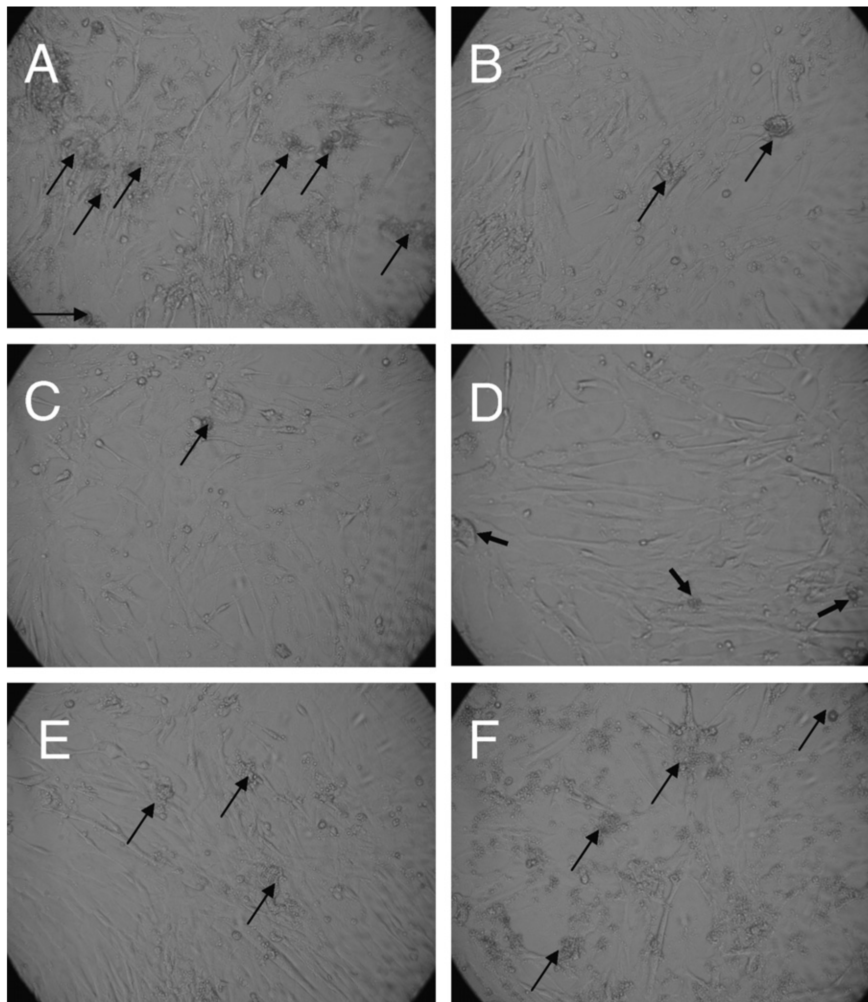

Fig. 1. The effects of adding soybean isoflavone (SI) at concentrations of 0 (A), 12.5 (B), 25 (C), 50 (D), 75 (E) and 100 (F) μM on morphology of chick skeletal cells cultured for 48 h. The oxidative damage was introduced by adding 80 μM H_2O_2/$FeSO_4$ to the media. The addition of SI prevented the morphological disruption of the cells caused by H_2O_2/$FeSO_4$. The arrows indicate disrupted cells.

Table 1
The effects of soybean isoflavone (SI) on proliferation of chick skeletal muscle cells and biochemical indices in supernatant of the medium for culture of chick skeletal muscle cells

	SI concentrations (μM)					
	0	12.5	25	50	75	100
Proliferation of chick skeletal muscle cells						
Absorbance	0.057 ± 0.004	$0.084 \pm 0.003^{**}$	$0.091 \pm 0.003^{**}$	$0.092 \pm 0.002^{**}$	$0.086 \pm 0.003^{**}$	$0.072 \pm 0.002^{*}$
Biochemical indices						
T-SOD[a] (U/ml)	2.26 ± 0.059	2.24 ± 0.050	$2.65 \pm 0.103^{**}$	$2.56 \pm 0.076^{*}$	$2.56 \pm 0.044^{*}$	$2.53 \pm 0.094^{*}$
GSH-px[b] (IU)	807 ± 234	$1214 \pm$ s221	$1539 \pm 54.6^{*}$	973 ± 255	866 ± 171	748 ± 117
CAT[c] (U/ml)	4.99 ± 0.197	5.38 ± 0.275	5.90 ± 0.339	5.22 ± 0.770	$7.45 \pm 0.484^{**}$	$7.44 \pm 0.514^{**}$
T-AOC[d] (U/ml)	3.36 ± 1.47	4.82 ± 1.29	5.91 ± 0.567	4.83 ± 0.875	3.68 ± 0.524	5.45 ± 1.30
CK[e] (IU/l)	323 ± 78.7	360 ± 72.0	370 ± 98.3	155 ± 23.7	$124 \pm 37.4^{**}$	$128 \pm 19.6^{**}$
LDH[f] (IU/l)	1385 ± 387	2051 ± 377	2023 ± 786	2220 ± 366	1547 ± 417	1736 ± 348

The oxidative damage to the cells was introduced by adding 80 μM $H_2O_2/FeSO_4$ to the media. The proliferation, as indicated by the absorbance at 550 nm, was determined by the MTT method with eight replicates. Each treatment had six replicates for biochemical indices. Values are means ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control (0 μM SI).

[a] T-SOD: total superoxide dismutase.
[b] GSH-px: glutathione peroxidase.
[c] CAT: catalase.
[d] T-AOC: total antioxidative capacity.
[e] CK: creatine kinase.
[f] LDH: lactate dehydrogenase.

Early studies suggested that SI is an inhibitor of the protein kinase that inhibits the activation of receptor tyrosine phosphorylation of epidermal growth factor, inducing DNA synthesis for cell proliferation *in vitro* and arresting the cell cycle progression. SI inhibit the cell proliferation and growth of aortic smooth muscle cells in human (Dubey et al., 1999) and in stroke–prone spontaneously hypertensive rats (Pan, Ikeda, Takebe, & Yamori, 2001). In contrast, we found that SI prevented morphological disruption of the SMC induced by oxidants, $H_2O_2/FeSO_4$, and promoted SMC proliferation. High doses of genistein cause primary cultured neuron death (Linford, Yang, Cook, & Dorsa, 2001). But a pre-treatment of the HCN1-A cell with 50 μM genistein could significantly protect the cells from the death induced by 100 μM and 1 mM *t*-BuOOH (Sonee, Sum, Wang, & Mukherjee, 2004). Genistein induced damage in human colon cells, and concentration–effect curves showed genistein to be genotoxic at lower concentrations (Pool-Zobel et al., 2000). The results suggested that the effect at the cellular level may be affected by the type of SI and concentrations (Cassidy, 2003; Liu et al., 2005). More research in this area is needed.

The present results demonstrate that the SI significantly enhanced the activities of T-SOD, GSH-px and CAT in the SMC media. These results are in agreement with the results of Cai and Wei (1996). In addition, Khan and Sultana (2004) reported that SI may return the lower levels of antioxidant enzymes (including GSH-px and CAT) induced by ferric nitrilotriacetate to normal levels in renal cells of Wistar rats. A possible contributory mechanism to antioxidant activity of isoflavonoids might be their ability to stabilize membranes by decreasing membrane fluidity. It has been suggested that isoflavonoids, similarly to cholesterol and tocopherol, partition into the hydrophobic core of the membrane and cause a dramatic decrease in lipid fluidity in this region of the membrane. Localization of isoflavonoids into membrane interiors and increased restrictions of the fluidity of membrane components could sterically hinder diffusion of free radicals and thereby decrease the kinetics of free radical reactions. SI has established antioxidant activity that might have counted against the oxidant effect of $FeSO_4/H_2O_2$ by scavenging free radicals.

Creatine kinase activity is a biochemical marker for muscle damage. The leak of CK from the muscle following damage can interrupt energy metabolism by decreasing the ability to generate ATP and phosphocreatine stores (Bessman & Carpenter, 1985). Loss of CK can also weaken muscle structure because it helps to form the tight lattice in the M-region of sarcomeres, and deteriorates the stability of contracting filaments. Persky et al. (2000) reported that estrogen decreases cumulative efflux of CK in ovariectomized soleus, which was in agreement with our results that SI evidently down-regulated the secretion of CK by the SMC into the media. The result suggests that SI may protect the cell membrane from CK leaking against oxidative damage.

In conclusion, we propose that possible mechanisms of chemopreventive effects of soybean isoflavones are: (1) induction of various antioxidant enzymes coupled with enhancement of detoxification processes and, (2) significant reduction of the level of CK secretion, which protects the cell membrane against oxidative damage. The results of the present study also indicate that the isoflavone had a beneficial effect on cell proliferation and antioxidant activities in chick skeletal muscle cells. However, further research is required to study the optimal amount and the

effects of SI on oxidative stability and shelf life of chick meat.

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