



Effect of Genistein on Antioxidative Defence System and Membrane Fluidity in Chick Skeletal Muscle Cells*

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ABSTRACT : This study was conducted to investigate the protective effect of genistein on the antioxidative defence system and membrane fluidity in chick skeletal muscle cells after supplementation with 0, 20, 40, and 80 $\mu\text{mol/L}$ genistein in 50 $\mu\text{mol/L}$ $\text{FeSO}_4/\text{H}_2\text{O}_2$ treated cells for 24 h. Genistein supplementation recovered the decreased activity of total superoxide dismutase induced by $\text{FeSO}_4/\text{H}_2\text{O}_2$, significantly increased glutathione peroxidase activity ($p < 0.05$) and decreased malondialdehyde production ($p < 0.05$). The treatment of 80 $\mu\text{mol/L}$ genistein in $\text{FeSO}_4/\text{H}_2\text{O}_2$ treated cells decreased the secretion of creatine kinase ($p < 0.05$). Fluorescence polarization values and microviscosities observed with $\text{FeSO}_4/\text{H}_2\text{O}_2$ treated cells were significantly higher than those observed with no $\text{FeSO}_4/\text{H}_2\text{O}_2$ treated cells. The addition of 80 $\mu\text{mol/L}$ genistein improved the increased fluorescence polarization value ($p < 0.05$) caused by $\text{FeSO}_4/\text{H}_2\text{O}_2$ treatment. The microviscosity value was significantly decreased by adding genistein ($p < 0.05$). In conclusion, genistein protected skeletal muscle cells from oxidative damage by improving antioxidative status and membrane fluidity. (**Key Words :** Genistein, Lipid Peroxidation, Membrane Fluidity, Antioxidation, Chick Skeletal Muscle Cells)

INTRODUCTION

In meat foods, previous studies have implicated the oxidation of polyunsaturated membrane lipids occurs during the development of off-flavors, loss of fresh meat color (Faustman et al., 1989), and forms the potentially harmful lipid oxides (Monahan et al., 1994). To effectively control lipid oxidation in meat foods at the initiation stage, the natural antioxidants, such as soybean isoflavone (Jiang et al., 2007), extracts of rosemary and sage (Lopez-Bote et al., 1998) or tea catechins (Tang et al., 2001), have been studied to maintain meat quality by reducing lipid peroxidation of meat products on shelf.

The isoflavone genistein is one of the most abundant polyphenolic compounds naturally present in soybeans, soy products and cereals (Anthony et al., 1998; Liggins et al., 2002). It has been proposed that the protective effect exerted by genistein against chronic vascular diseases and early atherogenic events could be related to its antioxidant

properties (Kapiotis et al., 1997). It has also been demonstrated that genistein inhibits lipid peroxidation induced *in vitro* by several pro-oxidant agents on model and natural membranes (Jha et al., 1985), in cultured cells (Guo et al., 2002; Ho et al., 2003) and in low density lipoproteins (Kerry et al., 1998; Wilson et al., 2002). Record et al. (1995) provided the evidence that genistein was an effective scavenger of H_2O_2 in liposomes. Thus, it is possible that genistein may protect chicken skeletal muscle cells against oxidation. Therefore, the objectives of this study were to determine the protective properties of genistein in chick skeletal muscle cells (SMC). The change in sideways fluidity of membrane molecules has become one of the symbols of cell transformation. Free radical peroxidation of unsaturated lipids in biomembranes was found to disrupt the various important structural and protective functions associated with biomembranes (Arora et al., 2000). Free radical production and lipid peroxidation affected membrane fluidity in primary rat hepatocytes (Sergent et al., 2005). In the present study, the effect of genistein on the membrane fluidity was also investigated by fluorescence polarization using 1-6-Diphenyl-1,3,5-hexatriene (DPH) as the probe in chick SMC. The results may help to understand the mechanism of the antioxidative function of genistein.

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Hopefully, the results would add to the background information concerning the use of genistein as a natural antioxidant in meat industry.

MATERIALS AND METHODS

Chemicals

Genistein (C₁₅H₁₀O₅), Dimethylsulfoxide (DMSO), and DPH were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA).

Cell culture and treatment

The chicken SMC were prepared from freshly dissected leg muscle of 20-day-old chick embryo as described by Matsuda et al. (1983). The muscles from both legs were carefully collected, and periadventitial fat and connective tissue were removed. The muscle was spliced to flat pieces about 4 mm², and incubated in a solution of 0.2% (w/v) type I collagenase (Sigma-Aldrich, St. Louis, MO) in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Invitrogen corporation, Shanghai, China) at 37°C for 2 h on a shaker with the speed of 180 rpm. The chicken SMC were cultivated in DMEM/F₁₂ containing 10% (v/v) fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin in 30 mm×60 mm culture glass dishes. The media were then maintained in a humidified incubator at 37°C and supplied with 5% CO₂ for 48 h. The cells were then transferred using 0.05% trypsin (Sigma-Aldrich, St. Louis, MO, USA) to serum-free DMEM. For biochemical measurements, 3 ml of the cell suspension was added into each well of 6-well microtiter plates. There were 6 replicate wells per treatment. The genistein was diluted in DMSO and added to the cell cultures to reach 0, 20, 40, and 80 µmol/L. The cells were treated with 50 µmol/L of ferrous ions (as FeSO₄·7 H₂O) and 50 µmol/L H₂O₂ to induce oxidative damage. The control cells were similarly treated with the same amount of DMSO (0.025% (v/v)).

Lipid peroxidation

The amount of lipid peroxidation was determined by measuring the release of the breakdown product malondialdehyde (MDA) into the medium after 24 h of incubation with genistein. The content of MDA was determined after reaction of cell culture supernatants with the enzymatic-colorimetric procedure (MDA kit, TBA Method; Nanjing Jiancheng Insititute of Bioengineering, Nanjing, China). Measurements were compared with untreated control cells.

Biochemical determinations

After the supplementation with 0, 20, 40, and 80 µmol/L genistein in 50 µmol/L FeSO₄/H₂O₂ treated cells for 24 h, the supernatant of the cell culture medium was taken for

analysis of activities of the total superoxide dismutase (T-SOD), glutathione peroxidase (GSHPx), and catalase (CAT) using colorimetric methods with a spectrophotometer (Biomate 5, Thermo Electron Corporation, Rochester, NY, USA). The assays were conducted using the assay kits (T-SOD kit, GSHPx kit, and CAT kit) purchased from Nanjing Jiancheng Insititute of Bioengineering (Nanjing, Jiangsu, China) and the procedures accordingly.

As indicators of cell cytotoxicity, the activities of creatine kinase (CK) and lactate dehydrogenase (LDH) in the cell culture supernatants were estimated 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).

Cell membrane fluidity

Membrane fluidity of chicken SMC was determined by the methods of Fuchs et al. (1975) after the cells had been incubated for 24 h in the presence or absence of genistein (0, 20, 40, and 80 µmol/L). Cell suspensions were prepared with a cell concentration of 4×10⁵ per ml in 3 ml of phosphate buffered solution (PBS). The fluorescent probes DPH and 4-trimethylammonium-DPH, which dissolved in acetone and dimethylformamide respectively (to 2×10⁻³ M), were added to separate suspensions to a final concentration of 2×10⁻⁶ M. The cells were labeled for 60 min and washed with PBS. Treatment for 3 min with 0.02% EDTA in phosphate buffer was immediately followed by addition of PBS. The cells were collected, centrifuged, and resuspended in PBS. Microscopic examination revealed no damage to the cells. Fluorescence anisotropy readings were made immediately for 4-trimethylammonium-DPH and after 30 min incubation for DPH using a spectrofluorometer (RF-5400, SHIMADZU, Japan) with polarizing filters. The mean of four readings was taken at 37°C with an excitation wavelength of 360 nm and an emission wavelength of 430 nm. The fluorescence polarization value, *p*, was calculated from Eq. 1 below. *I_{VV}* and *I_{VH}* are the intensities measured in directions parallel and perpendicular to the vector of the vertically polarized exciting light, *I_{HH}* and *I_{HV}* are the intensities measured in directions parallel and perpendicular to the vector of the horizontally polarized exciting light, and *G* is the grating correction factor:

$$P = (I_{VV} - GI_{VH}) / (I_{VV} + GI_{VH}), \quad G = I_{HV} / I_{HH} \quad (1)$$

Microviscosities were calculated following the procedure. This method is based on the Perrin Eq. 2:

$$r_0/r = 1 + C(r) \cdot Tr/\eta \quad (2)$$

for rotational depolarization of a nonspherical fluorophore. Here, *r* and *r₀* are the measured and limiting

Table 1. The effects of genistein on activities of T-SOD, GSHPx, CAT, CK, and LDH in supernatant of the medium for culture of chick skeletal muscle cells

Biochemical indices	FeSO ₄ /H ₂ O ₂ (μmol/L)				
	0	50	50	50	50
	Genistein concentrations (μmol/L)				
	0	0	20	40	80
T-SOD (U/ml)	8.83±0.490 ¹	4.35±0.680*	8.83±0.631 ^a	8.70±0.520 ^a	9.09±0.253 ^a
GSHPx (U/ml)	35.77±5.864	35.17±3.508	56.48±2.482* ^a	60.11±1.175* ^a	63.39±2.811* ^a
CAT (U/ml)	1.43±0.091	1.26±0.087	1.43±0.033	1.28±0.094	1.15±0.094
CK (U/L)	275.43±62.137	401.68±25.996	368.12±37.1712	321.22±55.291	225.10±22.389 ^a
LDH (U/L)	644.93±111.928	1,711.41±174.378*	1,684.91±337.051*	1,383.87±56.850*	1,187.01±91.931*

The oxidative damage was introduced by adding 50 μmol/L FeSO₄/H₂O₂ in the media.

¹ Values are means±SE for six replicates.

* p<0.05 vs. controls with no treatment, ^a p<0.05 vs. cells treated with 50 μmol/L FeSO₄/H₂O₂ only.

fluorescence anisotropies ($r_0 = 0.362$), T is the absolute temperature, and r is the excited state lifetime calculated from the measured relative fluorescence intensities at each temperature and a T_0 value of 11.4 nsec. $C(r)$ has a value of $8.6 \pm 0.4 \times 10^5$ poise deg⁻¹ sec⁻¹ and is a molecular shape parameter of the fluorophore; and η , the viscosity of the medium opposing the rotation of the fluorescent probe around its long axis, was calculated.

Statistical analysis

Data were indicated as means±SE. The effects of genistein at the various concentrations were compared with that of control by One-way analysis of variances using a computing software SAS (v6.12, SAS Institute Inc., Cary, NC, USA). Statistical significance was set at p<0.05.

RESULT

Antioxidant enzyme activities

Activities of the antioxidative enzymes, T-SOD, GSHPx

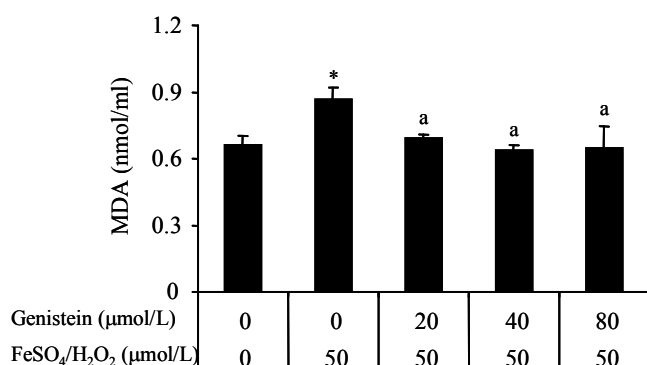


Figure 1. Formation of malondialdehyde (MDA) in chick skeletal muscle cells. Cells were incubated with the indicated concentrations of genistein, and with 50 μmol/L FeSO₄/H₂O₂ or none for 24 h. The amount of MDA released into the medium was determined. The results are expressed as means±SE, n = 6, * p<0.05 vs. controls with no treatment; ^a p<0.05 vs. cells treated with 50 μmol/L FeSO₄/H₂O₂ only.

and CAT in supernatant of the media from SMC culture are presented in Table 1. Exposed in the media containing 50 μmol/L FeSO₄/H₂O₂, the activity of T-SOD was significantly decreased by 50.74% (p<0.05) with respect to controls with no treatment, however, adding genistein at concentrations of 20, 40, and 80 μmol/L all recovered the declined activity of T-SOD by an inclusion of 50 μmol/L FeSO₄/H₂O₂ (p<0.05). Genistein treatment enhanced GSHPx activity in FeSO₄/H₂O₂ treated cell media (p<0.05, at 20 μmol/L by 54.90%, at 40 μmol/L by 67.10%, at 80 μmol/L by 80.24%, respectively). Addition of Genistein at the various concentrations had no significant effect on CAT activity (p>0.05).

Lipid peroxidation

The extent of lipid peroxidation was determined by measuring the release of the breakdown product MDA into the medium (Figure 1). Treatment with 50 μmol/L FeSO₄/H₂O₂ increased MDA levels by 32% as compared to controls (p<0.05). In cells supplemented with genistein, MDA production decreased with respect to cells treated with 50 μmol/L FeSO₄/H₂O₂ only (p<0.05).

Membrane damage by oxidants and membrane fluidity

Activities of CK and LDH in supernatant of the media from SMC culture are presented in Table 1. Exposed in the media containing FeSO₄/H₂O₂, genistein supplementation at 80 μmol/L significantly decreased CK secretion by about 44% (p<0.05). FeSO₄/H₂O₂ treatment significantly increased LDH activity in comparison with control cell media (p<0.05). Although adding genistein reduced LDH activities with a manner of dose response in the oxidant-treated cells, the reductions were not significant (p>0.05).

The fluorescence polarization value, p , and microviscosity, η of chick skeletal muscle cell membrane are shown in Figure 2. From the data, it was found that FeSO₄/H₂O₂ significantly increased p and η value (p<0.05). As compared to the FeSO₄/H₂O₂ treated cells, the addition

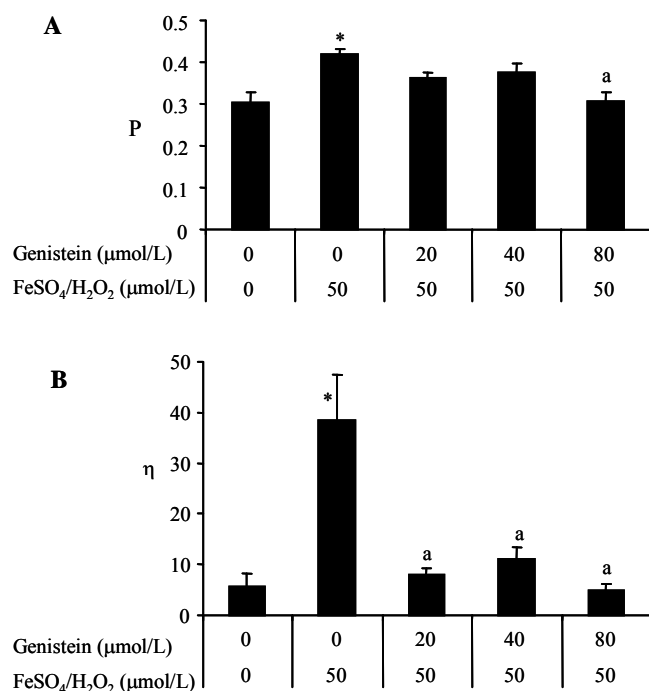


Figure 2. The fluorescence polarization value, p , and microviscosity, η of chick skeletal muscle cell membrane. (A) Cells were treated with the indicated concentrations of genistein, and with 50 $\mu\text{mol/L}$ $\text{FeSO}_4/\text{H}_2\text{O}_2$ or none for 24 h. The fluorescence polarization value, p of chick skeletal muscle cell membrane was determined, values are means \pm SE, $n = 6$, * $p < 0.05$ vs. controls with no treatment; ^a $p < 0.05$ vs. cells treated with 50 $\mu\text{mol/L}$ $\text{FeSO}_4/\text{H}_2\text{O}_2$ only. (B) Cells were incubated with the indicated concentrations of genistein, and with 50 $\mu\text{mol/L}$ $\text{FeSO}_4/\text{H}_2\text{O}_2$ or none for 24 h. The microviscosity, η of chick skeletal muscle cell membrane was determined, values are means \pm SE, $n = 6$, * $p < 0.05$ vs. controls with no treatment; ^a $p < 0.05$ vs. cells treated with 50 $\mu\text{mol/L}$ $\text{FeSO}_4/\text{H}_2\text{O}_2$ only.

of genistein decreased p value when its concentration reached 80 $\mu\text{mol/L}$ ($p < 0.05$). The η value was significantly decreased by adding genistein at concentrations of 20, 40, and 80 $\mu\text{mol/L}$, and the decline was 79.23% ($p < 0.05$), 71.15% ($p < 0.05$), and 87.53% ($p < 0.05$), respectively.

DISCUSSION

Accumulating evidence suggests that reactive oxygen species and their subsequent modification of macromolecules (such as DNA, lipid, and protein) play an important role in cytotoxicity, genotoxicity, and carcinogenic processes (Frenkel, 1975; Sun et al., 1990). Oxidative stress decreased activities of antioxidant enzymes and increased concentration of MDA in plasma and liver of weanling pigs (Yuan et al., 2007). Isoflavones have multiple hydroxyl group(s) to act as effective antioxidants by donating a hydrogen atom(s) from their phenolic hydroxyl

group(s) to peroxyradicals (Tikkanen et al., 1998). Liu et al. (2005) reported that soy isoflavone extract showed a strong *in vitro* antioxidant activity using the Rancimat induction time method. Genistein had been found to suppress H_2O_2 production by 12-O-tetradecanoylphorbol-13-acetate-stimulated human polymorphonuclear leukocytes and HL-60 cells in a dose-dependent manner over the concentration range of 1-150 μM concentrations (Wei et al., 1993). The results of this experiment showed that genistein improved the activities of T-SOD and GSHPx in the media of chick SMC. This was in agreement with the results in cultured chicken testicular cells by Mi et al. (2007). In addition, Choi et al. (2003) observed significant increases of SOD in genistein-treated (50-100 μM) RAW 264.7 macrophages. Moreover, Khan and Sultana (2004) reported that isoflavone may return the lower levels of GSHPx and CAT induced by ferric nitrilotriacetate to normal levels. Antioxidant enzymes are capable of eliminating reactive oxygen species and products of lipid peroxidation, thereby protecting cells and tissues from oxidative damage. The antioxidant enzymes include SOD, CAT and GSHPx (Hodgson et al., 1979; Aebi et al., 1984; Ursini et al., 1995). The GSHPx and CAT convert H_2O_2 to H_2O and the SOD catalyze the dismutation of the superoxide radical anion. Hence, the recovered activities of antioxidant enzymes indicated that genistein addition improved ability to detoxify H_2O_2 and the superoxide anion induced by $\text{FeSO}_4/\text{H}_2\text{O}_2$ in the medium for culture of chick SMC. These results also suggested that genistein was able to enhance the antioxidative property against oxidative damage to the cells.

Reactive oxygen species can initiate lipid peroxidation and DNA damage that leading to mutagenesis, carcinogenesis, and cell death, if the antioxidant system is impaired (Devi et al., 2000). The present results showed that $\text{FeSO}_4/\text{H}_2\text{O}_2$ treatment increased the MDA production. This suggested that lipid peroxidation in cells enhanced by $\text{FeSO}_4/\text{H}_2\text{O}_2$. On the contrary, the formation of MDA in chick SMC media was significantly ($p < 0.05$) decreased by genistein addition (Figure 1). These results are in agreement with the previous study (Foti et al., 2005) in oxidized human primary lymphocytes treated with 2.5 $\mu\text{mol/L}$ of genistein for 24 h. This may be attributed to the vital role of isoflavones as antioxidant factors. Genistein are protective antioxidants reducing the formation of radicals and reactive oxygen by decomposition of hydrogen peroxide without generating radicals, by quenching active singlet oxygen, and by trapping and quenching radicals before they reach a cellular target (Fran et al., 2000). This finding suggested that genistein has antioxidant activities and may be needed to protect cells against lipid peroxidation.

The activity of CK 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 store phosphocreatine (Bessman et al., 1985). Loss of CK can also weaken muscle structure because it helps form the tight lattice in the M-region of sarcomeres, and deteriorate 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 genistein evidently down-regulated the secretion of CK by the SMC into the media. The result suggests that genistein may protect the SMC against oxidative damage by inhibiting CK leaking.

The stretching, vibration, and side diffusion of the lipid molecules in the cell membrane contributed to the membrane lipid fluidity, which plays a very important role in maintaining the normal physiological functions of cells. A number of reasons have been advanced to explain the decrease in membrane fluidity associated with membranal lipid oxidation (Chen et al., 2002). It has been suggested that isoflavones can protect lipid membranes from oxidants *in vitro* (Arora et al., 1998; Arora et al., 2000). The fluorescence polarization value and microviscosity of chick skeletal muscle cell membrane observed in this study indicated that membrane lipid fluidity of chick SMC was decreased after FeSO₄/H₂O₂ treatment. However, the addition of genistein could improve membrane lipid fluidity of cells. Results from this study suggest that genistein has the beneficial effects on cell membrane by improving membrane fluidity.

In summary, the present *in vitro* studies demonstrated that genistein inhibits lipid oxidation and CK secretion, and improves membrane fluidity in chick SMC. In addition, the results support the hypothesis that genistein ameliorates FeSO₄/H₂O₂-mediated inhibition of the activities of antioxidant enzymes. Thus, genistein acting as a dietary antioxidant may have great benefit for prevention of chick meat oxidation. Further research is required to study the effects of soy isoflavones on oxidative stability and shelf life of chick meat *in vivo*.

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