J Food Sci Nutr
Vol 14, p 37~42 (2009)
DOI: 10.3746/jfn.2009.14.1.037

J Food Science and Nutrition

Protective Activity of Seolitae Chungkukjang Added with Green Tea against Cellular Oxidative Stress induced by AAPH

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Abstract

The protective activity of seolitae chungkukjang added with green tea against oxidative stress was investigated under the cellular systems using LLC-PK$_1$ cells. The treatment of 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH) showed increase in lipid peroxidation, and decrease in endogenous antioxidant enzymes activity and cell viability. However, the methanol extract of seolitae chungkukjang inhibited lipid peroxidation by 58.3%, and increased cell viability up to more than 60%. In addition, it enhanced superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. Seolitae chungkukjang improved oxidative stress-induced cellular injury through the radical scavenging activities. In particular, the addition of green tea in seolitae chungkukjang showed stronger effect against oxidative stress induced by AAPH. The more addition of green tea resulted in the greater antioxidative effect through elevation in activities of SOD and GSH-Px, and inhibition of lipid peroxidation, eventually leading to increase in cell viability. Theses results suggested that seolitae chungkukjang added with green tea have protective effects from cellular oxidative damage and could be considered as an application for the development of chungkukjang with functionality.

Key words: seolitae, chungkukjang, green tea, 2,2'-azobis (2-aminopropane) dihydrochloride, LLC-PK$_1$

INTRODUCTION

A well-designed *in vitro* model system is necessary to study the reactions of free radicals which are more complicated in biological systems. Oxidative stress is occurred by the numerous factors such as irradiation, redox decomposition by metal ions of hydroperoxides or hydrogen peroxide, and thermal decomposition of free radical initiators including peroxides, hyponitrites and azo compounds. To generate free radicals at a controlled and well-defined rate, azo compounds are widely employed *in vitro* and *in vivo* on the protective effects of antioxidants as well as for the actions of free radicals upon biological molecules (1-3). 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH), one of the hydrophilic azo compounds, generates free radicals reacting with oxygen molecules rapidly to yield peroxyl radicals. The peroxyl radicals attack other lipid molecules to form lipid hydroperoxide and newly formed lipid radicals. This reaction takes place repeatedly with resultant attacks upon various biological molecules, and induces physiochemical alterations and cellular damage (4). Therefore, an AAPH intoxication experiment may be a promising assay system for the biological activities of antioxidants. In addition, LLC-PK$_1$, a renal-tubular epithelial cell line, is susceptible to oxidative stress, resulting in cell death or injury. It was reported that AAPH led to the decreased viability of LLC-PK$_1$ renal epithelial cells (5). In AAPH-induced cell injury and peroxidation, scavenging of lipid peroxyl radicals seems to play a considerable part in antioxidative activity (6).

Chungkukjang is a Korean traditional fermented soybean, which has been widely consumed foods as a protein source. Chungkukjang is a good source of digestible protein and various bioactive compounds, since soybean protein is digested into peptones, peptides and amino acids. In addition chungkukjang has various physiological effects that are beneficial to human health, such as the prevention of thrombosis, anti-cancer, high blood pressure-lowering, and serum cholesterol-lowering effects (7-9). To take advantage of health benefits of chungkukjang, efforts to develop chungkukjang as a functional food are currently in progress.

Previously we reported that seolitae chungkukjang exerted the stronger radical scavenging effect, thus in the present study we used seolitae instead of soybean as the major material for chungkukjang (10). Furthermore, the addition of green tea showed the greater *in vitro* antioxidative effects via radical scavenging activities and ex-

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hibited the similar sensory preference compared to soybean chungkukjang (10). Therefore the present study focused on the protective activity of green tea added seolitae chungkukjang under a cellular systems using LLC-PK_1 renal epithelial cells susceptible to oxidative stress.

MATERIALS AND METHODS

Materials

Seolitae was purchased from Dongnae local market (Busan, Korea) and green tea was obtained from Amorepacific Corp. (Jincheon, Korea). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY, USA). 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH), glutathione peroxidase kit were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade.

Preparation of samples

The preparation of chungkukjang and the extraction process by MeOH were followed as described previously (10). The extracts were concentrated using a rotary evaporator and they were dissolved in dimethylsulfoxide (DMSO) diluted with PBS that did not show cytotoxicity in LLC-PK_1 cell in preliminary test.

Cell culture

LLC-PK_1, porcine renal epithelial cell, was obtained from ATCC (Manassas, VA, USA). The LLC-PK_1 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 incubator with 5% FBS and DMEM supplemented with 1% penicillin-streptomycin.

Cell viability assay

Cell viability was determined by an MTT colorimetric assay (11). After confluence had been reached, LLC-PK_1 cell were seeded at 1×10^4 cells/mL in 96-well plate and incubated for 2 hrs before treatment of 10 mM AAPH. After 24 hrs, various concentrations of sample (100, 250, and 500 μg/mL) were treated for 24 hrs. After that, 100 μL of MTT (5 mg/mL) solution was added to each well followed by 4 hrs incubation at 37°C. The MTT solution was removed from the plate and the resultant formazan crystal were solubilized with 100 μL of DMSO. The absorbance of each well at 540 nm was determined using an ELISA microplate reader.

Lipid peroxidation analysis

Lipid peroxidation was performed by quantifying thiobarbituric acid-reactive substances (TBARS) with slight modifications (12). LLC-PK_1 cell were seeded at 1×10^4 cells/mL in 96-well plate and incubated for 2 hrs before treatment of 10 mM AAPH. After 24 hrs incubation with 10 mM AAPH, various concentrations of sample were treated for 24 hrs. One aliquot of medium was mixed with 1 mL of 1% TBA aqueous solution and 1 mL of 25% TCA, and heated at 95°C for 20 min. After cooling down, the mixture was shaken vigorously with 3 mL of n-butanol, and centrifuged at $4000\times g$ for 30 min. The supernatant was measured spectrophotometrically at 532 nm. The value was expressed as nmole of malondialhdehyde (MDA) per mg protein.

Antioxidative enzyme assay

After 2 hrs pre-incubation of cells (5×10^4 cells/mL), 10 mM AAPH was added and incubated for 24 hrs. Various concentrations of sample (100, 250, and 500 μg/mL) were treated and incubated for 24 hrs. After removal of media, cells were washed twice with PBS solutions. Cell suspension was sonicated on ice three time for 5 sec, and centrifuged at $10{,}000\times g$ for 20 min. The supernatants were used for determination of antioxidant enzyme activities. Superoxide dismutase (SOD) activity was measured as the methods of Ewing and Janero (13). One unit (U) of SOD activity was defined as an amount of enzyme required to give 50% inhibition of the initial rate of pyrogallol reduction. Glutathione peroxidase (GSH-Px) activity was determined using commercially available kit followed by the methods of Lawrence and Burk (14). One unit (U) of GSH-Px activity was an amount of enzyme required to oxidize 1 nmole of NADPH per min.

Statistical analysis

All statistical analyses were performed by SAS software (SAS Institute, Cary, NC, USA). $p<0.05$ was determined as statistically significant. All data were expressed as mean±standard deviation (n=5).

RESULTS AND DISCUSSION

To investigate the protective effects of chungkukjang ingredients against AAPH-derived cytotoxicity, we examined the effects of seolitae and green tea on lipid oxidation and cell viability of LLC-PK_1 cells. Lipid peroxidation was significantly increased from 0.203 nmol MDA/mg protein to 1.305 nmol MDA/mg protein, after treatment of 10 mM AAPH in LLC-PK_1 cells (Fig. 1). However, the addition of both seolitae and green tea extracts inhibited the MDA production in a dose-dependant manner. At the concentration of 100 μg/mL and 500 μg/mL of seolitae extract, the concentration of MDA were decreased by 41.5% and 55.5% compared to control. Although MeOH extract of soybean also showed the in-

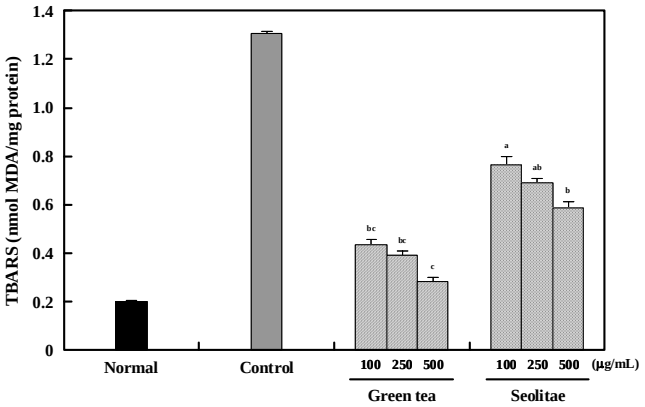

Fig. 1. Effect of MeOH extract from green tea, soybean and seolitae on TBARS generation in AAPH-treated LLC-PK$_1$ cells. Values are mean±SD. [a-c]Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

hibition of lipid peroxidation dose-dependantly, it had less inhibitory effect on MDA production than MeOH extract of seolitae ($p<0.05$; data not shown). MeOH extract of green tea showed better inhibitory effect of lipid peroxidation as decrease by 66.6% and 78.2% at the concentration of 100 μg/mL and 500 μg/mL.

The effects of seolitae and green tea on the cell viability were evaluated in LLC-PK1 cells treated with 10 mM AAPH through the generation of peroxy radicals. As shown in Fig. 2, cell viability was decreased to 34.5% after treatment of AAPH. However, by the protective effects of seolitae and green tea, MeOH extract of seolitae and green tea exerted increase in cell viability in a dose-dependant manner. At the concentration of 500 μg/mL, the cell viability was elevated to 62.9% (seolitae) and 95.8% (green tea), respectively. Green tea extract resulted in increase of cell survival against oxidative

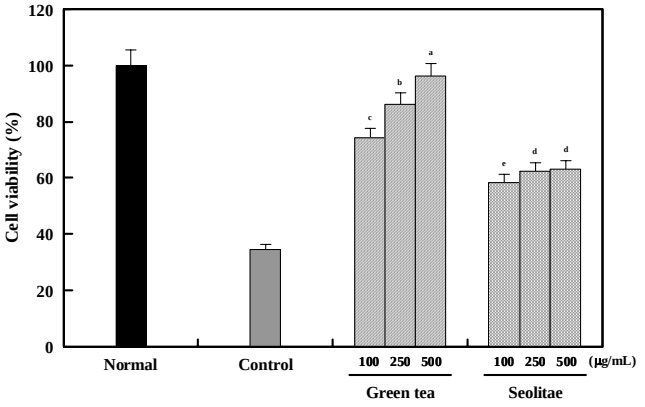

Fig. 2. Protective effect of MeOH extract from green tea, soybean and seolitae from AAPH-induced oxidative stress in LLC-PK$_1$ cells. Values are mean±SD. [a-e]Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

stress induced by AAPH.

From these results, chungkukjang was prepared using seolitae as a main ingredient and green tea as a minor ingredient. The preparation of chungkukjang was followed by the methods reported previously (10). According to the preparation methods, seolitae chungkukjang (SC), seolitae chungkukjang with 0.5% green tea (SCG0.5), seolitae chungkukjang with 2% green tea (SCG2) and seoliate chungkukjang with 5% green tea (SCG5) were prepared. After extraction with MeOH, protective effects of seolitae chungkukjang added with or without green tea were investigated.

Cells are protected from activated oxygen species by endogenous antioxidant enzymes such as SOD and GSH-Px. The effects of chungkukjang extract on SOD activities in AAPH-treated LLC-PK$_1$ cells were shown in Table 1. The treatment of 10 mM AAPH in LLC-PK$_1$ cells lowered SOD activity by 47.1% compared to untreated cells. After addition of seolitae chungkukjang extract, SOD activity was increased significantly. However, addition of green tea in seolitae chungkukjang had no effect on SOD activities until 500 μg/mL of seolitae chungkukjang extract added 2% green tea or all concentration of seolitae chungkukjang extract added 5% green tea was used.

GSH-Px activity in AAPH-treated LLC-PK$_1$ cells was significantly decreased to 62.57 U/mg protein compared to 104.46 U/mg protein in untreated cells (Table 2). Seolitae chungkukjang extracts elevated GSH-Px activ-

Table 1. Effect of chungkukjang extract on SOD activity in AAPH-treated LLC-PK$_1$ cells

Treatment (μg/mL)		SOD activity (U/mg protein)
Normal		6.48±0.28
AAPH-treated control		3.43±0.29
SC	100	4.51±0.16[c]
	250	4.83±0.85[c]
	500	4.84±0.07[c]
SCG0.5	100	4.80±0.05[c]
	250	4.81±0.04[c]
	500	4.85±0.05[c]
SCG2.0	100	4.61±0.18[c]
	250	4.46±0.11[c]
	500	5.84±0.14[b]
SCG5.0	100	5.78±0.15[b]
	250	5.98±0.39[ab]
	500	5.56±0.76[b]

SC, seolitae chungkukjang; SCG0.5, seolitae chungkukjang added with 0.5% green tea; SCG2.0, seolitae chungkukjang added with 2.0% green tea; SCG5.0, seolitae chungkukjang added with 5.0% green tea.
Values are mean±SD.
[a-c]Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

Table 2. Effect of chungkukjang extract on GSH-Px activity in AAPH-treated LLC-PK$_1$ cells

Treatment (μg/mL)		GSH-Px activity (U/mg protein)
Normal		104.46±7.49
AAPH-treated control		62.57±9.42
SC	100	62.84±9.90^{d}
	250	71.41±12.38^{cd}
	500	82.12±16.37^{c}
SCG0.5	100	89.80±1.72^{b}
	250	83.79±6.60^{bc}
	500	83.29±10.32^{c}
SCG2.0	100	88.55±4.95^{b}
	250	76.17±16.49^{cd}
	500	83.29±10.32^{c}
SCG5.0	100	80.50±4.50^{c}
	250	95.02±8.09^{ab}
	500	94.93±6.34^{ab}

SC, seolitae chungkukjang; SCG0.5, seolitae chungkukjang added with 0.5% green tea; SCG2.0, seolitae chungkukjang added with 2.0% green tea; SCG5.0, seolitae chungkukjang added with 5.0% green tea.
Values are mean±SD.
[a-d]Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

Table 3. Effect of chungkukjang extract on TBARS generation in AAPH-treated LLC-PK$_1$ cells

Treatment (μg/mL)		TBARS (nmol MDA/mg protein)
Normal		0.203±0.021
AAPH-treated control		1.305±0.040
SC	100	0.692±0.023^{a}
	250	0.603±0.032^{b}
	500	0.544±0.048^{c}
SCG0.5	100	0.738±0.024^{a}
	250	0.663±0.023^{ab}
	500	0.518±0.036^{c}
SCG2.0	100	0.713±0.021^{a}
	250	0.582±0.012^{b}
	500	0.422±0.008^{cd}
SCG5.0	100	0.679±0.010^{a}
	250	0.506±0.051^{c}
	500	0.340±0.015^{d}

SC, seolitae chungkukjang; SCG0.5, seolitae chungkukjang added with 0.5% green tea; SCG2.0, seolitae chungkukjang added with 2.0% green tea; SCG5.0, seolitae chungkukjang added with 5.0% green tea.
Values are mean±SD.
[a-d]Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

ities in a dose-dependant manner. GSH-Px activities at the concentration of 500 μg/mL in green tea-added group were 83.29 (SCG0.5), 83.29 (SCG2.0), and 94.93 (SCG5.0) U/mg protein, respectively. GSH-Px activities against oxidative stress induced by AAPH had been retained by more than 80% in green tea-added groups.

Inhibitory effects of chungkukjang extracts on the lipid peroxidation were performed by the measurement of lipid peroxide induced by AAPH via peroxy radical generation, which were shown in Table 3. The treatment of chungkukjang extracts inhibited MDA production in LLC-PK$_1$ cells. Comapred to 1.305 nmol MDA/mg protein of AAPH-treated control, TBARS generation was decreased to 0.544 (SC), 0.518 (SCG0.5), 0.422 (SCG2.0), and 0.340 (SCG5.0) at the concentration of 500 μg/mL. The amount of MDA was decreased dose-dependantly as the amount of green tea added was increased.

Cell viability of AAPH-treated LLC-PK$_1$ cells using MTT assay resulted in Table 4. In all groups, cell survivals were increased more than 60%. Dose-dependant increase in cell viability was not observed, but cell survival was elevated as the amount of green tea in seolitae chungkukjang. At 500 μg/mL of SCG5.0, cell viability was retained by 76.8%.

Seolitae is a kind of black soybean which contained considerable amount of anthocyanin in the seed coat (15). It was reported that anthocyanin played important roles as dietary antioxidants in the prevention of oxidative damages and have several biological activities such as anticonvulsant, anticarcinogenic, antiatherosclerotic, and anti-inflammatory actions (16-21). Ghiselli et al. (22) reported that anthocyanin had high antioxidant activity through the peroxy radical scavenging. MeOH extract of seolitae chungkukjang showed higher inhibitory effect of lipid peroxidation and elevated cell viability

Table 4. Effect of chungkukjang extract on cell viability in AAPH-treated LLC-PK$_1$ cells

Treatment (μg/mL)		Cell viability (%)
Normal		100.0±3.8
AAPH-treated control		34.5±1.2
SC	100	60.2±3.1^{c}
	250	63.9±3.9^{c}
	500	62.0±2.2^{c}
SCG0.5	100	64.7±3.0^{bc}
	250	66.9±4.2^{b}
	500	66.8±0.8^{b}
SCG2.0	100	67.3±1.1^{b}
	250	69.4±5.2^{ab}
	500	70.6±6.1^{ab}
SCG5.0	100	72.1±2.5^{a}
	250	75.1±3.0^{a}
	500	76.8±2.8^{a}

SC, seolitae chungkukjang; SCG0.5, seolitae chungkukjang added with 0.5% green tea; SCG2.0, seolitae chungkukjang added with 2.0% green tea; SCG5.0, seolitae chungkukjang added with 5.0% green tea.
Values are mean±SD.
[a-c]Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

in LLC-PK_1 cell treated with AAPH than that of soybean (data not shown). In addition, the activities of endogenous antioxidant enzymes such as SOD and GSH-Px were elevated along with the treatment of seolitae chungkukjang extract. Anthocyanin in seolitae is considered to have protective potential from oxidative stress induced by AAPH under cellular oxidative damage.

The antioxidant activity of green tea is well-known by the suppression of the occurrence of lipid peroxidation in the biological systems (23-25). Phenolic compounds of green tea shows not only radical scavenging effects but also the inhibition of oxidative stress-induced apoptosis (26,27). As previously reported (10), the addition of green tea up to 5% in seolitae chungkukjang showed the lower off-flavor, the better overall taste compared to soybean chungkukjang, although the statistical significance has not been shown. In the present study, the addition of green tea to seolitae chungkukjang it elevated endogenous antioxidant enzymes and cell viability and suppressed lipid peroxidation in a dose dependant manner. As expected, protective effects in LLC-PK_1 cells were improved against oxidative damage induced by AAPH.

In conclusion, green tea added seolitae chungkukjang appeared to have a scavenging activity toward peroxy radicals generated from AAPH, and thereby increase cell viability and reduce MDA formation in LLC-PK_1 cells. It may be possible to apply seolitae and green tea as prospective materials for the development of chungkukjang with functionality.

ACKNOWLEDGEMENTS

This work was supported for two years by Pusan National University Research Grant.

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(Received March 4, 2009; Accepted March 13, 2009)