J Food Sci Nutr
Vol 13, p 90~94 (2008)
DOI: 10.3746/jfn.2008.13.2.090

J Food Science and Nutrition

Protective Effect of Chungkukjang from Sunchang Province against Cellular Oxidative Damage

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Abstract

The protective effect of chungkukjang from Sunchang province against oxidative stress was evaluated in the cellular system using LLC-PK$_1$ renal epithelial cells. The LLC-PK$_1$ cells showed decrease in cell viability and elevation in lipid peroxidation by the treatment with the generators of nitric oxide (NO) and superoxide anion (O_2^-) produced by sodium nitrouprusside and pyrogallol, respectively. However, the methanol extract of chungkukjang significantly inhibited cellular loss and lipid peroxidation in a dose-dependent manner; in particular K chungkukjang (KC) exerted the strongest protective effect. In addition, the protective effect of chungkukjang from 3-morpholinosydnonimine, as a source of peroxynitrite, with simultaneous generations of NO and O_2^-, was also studied. Treatment with chungkukjangs significantly preserved the cell viability and inhibited lipid peroxidation caused by SIN-1 with dose-dependence. The present study suggests that chungkukjang from Sunchang province, especially KC, would have protective potential from oxidative stress induced by free radicals under cellular oxidative damage.

Key words: chungkukjang, LLC-PK$_1$, nitric oxide, superoxide anion, peroxynitrite

INTRODUCTION

Reactive oxygen species (ROS), which include hydroxyl radical, superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2), play an integral role in the modulation of several physiological functions, but can also be destructive with excessive production. Similarly, over production of reactive nitrogen species (RNS) such as nitric oxide (NO), nitrite, and peroxynitrite ($ONOO^-$) can be potentially destructive, although they are physiologically necessary (1). In particular, $ONOO^-$ is very reactive and toxic oxidant formed by the reaction between NO and O_2^-. To alleviate the oxidative damage induced by ROS and RNS, antioxidants are promising agents. Researchers make efforts to find antioxidant without toxic side effects.

Chungkukjang is a Korean traditional fermented food made by fermenting soybean with *Bacillus natto* or *Bacillus subtilis* spp. Soybean protein in chungkukjang is digested to peptones, peptiedes and amino acids. Therefore, it is good source of digestable proteins and various bioactive compounds. Furthermore, chungkukjang comes of great interest among fermented food, since only a short time fermentation period is required compared to other products (2). Numerous studies have demonstrated that chungkukjang has the effects of thrombolysis (3-5), antihypertention (6), antimicrobiological activity (7) and antioxidative effect (8-12).

Although Sunchang province is famous for fermented foods including chungkukjang, the comparison of antioxidative activity has not been conducted yet. In the present study, we focused and evaluated the comparison of protective activity among chungkukjang from Shunchang province against NO, O_2^-, and $ONOO^-$-induced oxidative damage under a cellular system using LLC-PK$_1$ renal epithelial cells susceptible to oxidative stress.

MATERIALS AND METHODS

Materials

Sodium nitroprusside (SNP) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT) were obtained from Wako pure Chemical Industries, Ltd. (Osaka, Japan). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY, USA).

Preparation of sample

Three kinds of chungkukjang was obtained from Sunchang province. The MeOH extract of chungkukjang was prepared as follows; the freeze-dried chungkukjang was extracted by methanol (MeOH) at room temperature

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Table 1. Yield of MeOH extracts from chungkukjangs

Sample	Yield (%)
MC	32.2
HC	23.9
KC	19.3

MC: Moon Chungkukjang, HC: Hyang Chungkukjang, KC: Kim Chungkukjang

for 24 hrs and the MeOH extraction process was repeated 3 times. The extract was concentrated using a rotary evaporator and it was dissolved in dimethylsulfoxide (DMSO). Table 1 is the yield of MeOH extracts from chungkukjangs.

Cell culture

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

Cell viability analysis

Cell viability was determined using an MTT colorimetric assay (13). LLC-PK_1 cell seeded at 1×10^5/well in 96-well plates and pre-incubated for 2 hrs before treatment with SNP (1.2 mM), pyrogallol (1.2 mM) and SIN-1 (1 mM). After 24 hrs, various concentrations of sample (100, 250, 500, 750, 1000 μg/mL) were treated for 24 hrs. Thereafter, 100 μL of MTT (5 mg/mL) solution was added to the each well. After incubation for 4 hrs at 37°C, the MTT solution was removed from the plate. The resultant formazan crystals in the renal cells were solubilized with 100 μL of DMSO. The absorbance of each well was then read at 540 nm using a ELISA plate reader.

Lipid peroxidation analysis

Lipid peroxidation was measured by quantifying thiobarbituric acid-reactive substances, mainly malondialdehyde (MDA), formed during incubation using the thiobarbiturate-MDA adduct formation (14). One aliquot of medium was mixed with 1.5 mL of 0.67% TBA aqueous solution and 1.5 mL of 20% trichloroacetic acid, and boiled at 95~100°C for 45 min. The mixture was cooled with water and shaken vigorously with 3.0 mL of n-butanol. After the mixture was centrifuged at 4000× *g* for 10 min, the n-butanol layer was removed, and then the absorbance was measured on an ELISA. The product of the reaction between cell lysates and TBA was measured spectrophotometricly at 535 nm and values were expressed as nmole/mg protein.

Statistical analysis

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

RESULTS AND DISCUSSION

Chungkukjang is well known to have several biological effects such as thrombolysis, antihypertention, antimicrobiological effect and possible antioxidative activity (15). However, the research on antioxidative effect against cellular system has not been carried out. In this study, we used three kinds of chungkukjang. Sunchang province is famous for the production of soy and bean pastes, including chungkukjang. Therefore, we collected six kinds of chungkukjang in Sunchang and then, we chose the top three on the basis of the results of *in vitro* antioxidative effect, physicochemical properties and sensory evaluation.

NO is a widespread intra- and intercellular messenger and cytotoxin. The excessive production of NO participates in the pathology of inflammation, shock and injury to living tissue (16-19). In addition, it produces secondary active substances by reacting with oxygen and active oxygen species, resulting in induction of toxic effects (20-22). In the present study, to investigate the protective effect against NO, SNP was used and then the comparison of activity on cell viability and lipid peroxidation of chungkukjang was carried out. Table 2 shows the effect of MeOH extract from three kinds of chungkukjang against NO on cell viability of LLC-PK_1 renal epithelial cells. Cell viability of the control group was markedly decreased to 26.6% compared with 100% of the normal group. Three kinds of chungkukjang exerted increase in cell viability in a dose-dependant manner. At 1000 μg/mL, the cell viability was elevated to 68.7%

Table 2. Effect of MeOH extract from chungkukjang on viability of LLC-PK_1 cells treated with SNP

Treatment (μg/mL)	Cell viability (%)		
	MC	HC	KC
100	30.1±0.7^{e}	34.3±0.9^{e}	37.7±0.9^{e}
250	45.5±1.9^{d}	51.1±1.1^{d}	53.8±0.8^{d}
500	54.6±1.8^{c}	61.9±1.1^{c}	67.7±1.5^{c}
750	60.1±1.5^{b}	66.1±2.3^{b}	70.9±0.9^{b}
1000	68.7±1.1^{a}	71.0±1.3^{a}	76.0±1.0^{a}
SNP-treated control	26.6±1.5		
Normal	100.0±1.1		

MC: Moon Chungkukjang, HC: Hyang Chungkukjang, KC: Kim Chungkukjang.
Values are mean±SD.
$^{a-e}$Means with different letters are significantly different ($p<0.05$) from treatment concentration by Duncan's multiple range test.

Table 3. Protective effects of MeOH extract from chungkukjang on TBARS generation in SNP-treated LLC-PK$_1$ cells

Treatment (μg/mL)	MDA (nmole/mg protein)		
	MC	HC	KC
100	0.641±0.007[a]	0.620±0.008[a]	0.602±0.011[a]
250	0.591±0.009[b]	0.537±0.016[b]	0.529±0.017[b]
500	0.530±0.014[c]	0.490±0.006[c]	0.483±0.008[c]
750	0.335±0.016[d]	0.322±0.005[d]	0.316±0.005[d]
1000	0.328±0.020[d]	0.316±0.007[d]	0.299±0.010[e]
SNP-treated control	0.792±0.005		
Normal	0.209±0.010		

MC: Moon Chungkukjang, HC: Hyang Chungkukjang, KC: Kim Chungkukjang.
Values are mean±SD.
[a-e]Means with different letters are significantly different ($p<0.05$) from treatment concentration by Duncan's multiple range test.

Table 4. Effect of MeOH extract from chungkukjang on viability of LLC-PK$_1$ cells treated with pyrogallol

Treatment (μg/mL)	Cell viability (%)		
	MC	HC	KC
100	47.4±1.9[e]	51.9±1.0[e]	54.5±1.1[e]
250	54.6±1.0[d]	64.2±1.2[d]	65.4±1.1[d]
500	60.2±1.9[c]	68.3±0.8[c]	69.9±1.4[c]
750	67.3±1.4[b]	71.9±1.4[b]	76.6±0.9[b]
1000	83.4±1.5[a]	83.5±1.4[a]	85.4±1.1[a]
Pyrogallol-treated control	31.4±0.7		
Normal	100.0±0.9		

MC: Moon Chungkukjang, HC: Hyang Chungkukjang, KC: Kim Chungkukjang.
Values are mean±SD.
[a-e]Means with different letters are significantly different ($p<0.05$) from treatment concentration by Duncan's multiple range test.

(MC), 71.0% (HC) and 76.0% (KC), respectively. In addition, as shown in Table 3, NO led to the elevation of lipid peroxidation levels, from 0.209 nmole/mg protein to 0.792 nmole/mg protein. The treatment with chungkukjang decreased it dose-dependently. Among the chungkukjang, KC exerted the strongest effect. At a concentration of 1000 μg/mL, an 81% decrease (from 0.792 nmole/mg protein to 0.299 nmole/ mg protein) of lipid peroxidation was observed. From these results, we could confirm the protective activity of chungkukjang from NO-induced oxidative damage under cellular system.

For the evaluation of protective activity from O_2^-, pyrogallol was used. Superoxide anion generated by pyrogallol led to the loss of cell viability to 31.4% from 100% of non-treated cells, as shown in Table 4. However, the treatment with chungkukjang elevated cell viability in a dose-dependent manner. At the concentration of 1000 μg/mL, greater than 80% cell viability was observed in the all groups treated chungkukjang MeOH extracts. Table 5 shows the protective effect of chungkukjang from lipid peroxidation induced by O_2^-. Superoxide anion increased cellular lipid peroxidation from 0.220 nmole/mg protein to 0.892 nmole/mg protein. However, MeOH extract from chungkukjang inhibited the formation of MDA significantly and dose-dependently. In particular, KC showed the most effective activity. At 1000 μg/mL, it decreased lipid peroxidation from 0.892 nmole/mg protein to 0.306 nmole/mg protein (86% decrease). Similar to the result on NO, KC had the strongest protective effect against O_2^--induced cellular oxidative damage.

NO and O_2^- are known to rapidly react to form $ONOO^-$. $ONOO^-$ is a powerful oxidant inducing a wide array of tissue damage through lipid peroxidation, in-

Table 5. Protective effect of MeOH extract from chungkukjang on TBARS generation in pyrogallol-treated LLC-PK$_1$ cells

Treatment (μg/mL)	MDA (nmole/mg protein)		
	MC	HC	KC
100	0.872±0.011[a]	0.666±0.006[a]	0.662±0.013[a]
250	0.781±0.008[b]	0.519±0.008[b]	0.518±0.013[b]
500	0.530±0.011[c]	0.474±0.006[c]	0.480±0.009[c]
750	0.453±0.006[d]	0.366±0.007[d]	0.344±0.017[d]
1000	0.417±0.008[e]	0.319±0.010[e]	0.306±0.009[e]
Pyrogallol-treated control	0.892±0.007		
Normal	0.220±0.012		

MC: Moon Chungkukjang, HC: Hyang Chungkukjang, KC: Kim Chungkukjang.
Values are mean±SD.
[a-e]Means with different letters are significantly different ($p<0.05$) from treatment concentration by Duncan's multiple range test.

activation of enzymes and ion channels via protein oxidation and nitration to inhibit mitochondrial respiration (23-25). We used SIN-1 for the generation of $ONOO^-$. Against $ONOO^-$, the treatment of chungkukjang MeOH extract led to a significant increase in cell viability (Table 6). In particular, KC showed the strongest effect among three kinds of chungkukjang. The cell viability was increased to 81.2% by treatment with 1000 μg/mL from 21.0% of non-treated control. Furthermore, as shown in Table 7, $ONOO^-$ generated by SIN-1 increased formation of MDA as compared with normal group, whereas MeOH extract from chungkukjang led to a decline in the level. The protective effect was the strongest in the group treated with MeOH extract from KC. At the concentrations of 500 and 1000 μg/mL, MDA level was decreased to 0.312 nmole/mg protein (83% decrease) and 0.191 nmole/mg protein (99% decrease)

Table 6. Effect of MeOH extract from chungkukjang on viability of LLC-PK_1 cells treated with SIN-1

Treatment (μg/mL)	Cell viability (%)		
	MC	HC	KC
100	38.1±1.1[e]	46.9±0.6[e]	50.9±1.0[e]
250	51.7±0.9[d]	56.0±1.2[d]	60.7±1.6[d]
500	58.6±1.3[c]	62.0±3.5[c]	68.1±2.0[c]
750	67.1±1.2[b]	72.0±0.7[b]	77.2±2.3[b]
1000	70.4±1.2[a]	74.9±1.8[a]	81.2±2.0[a]
SIN-1-treated control	21.0±0.8		
Normal	100.0±1.4		

MC: Moon Chungkukjang, HC: Hyang Chungkukjang, KC: Kim Chungkukjang.
Values are mean±SD.
[a-e]Means with different letters are significantly different ($p<0.05$) from treatment concentration by Duncan's multiple range test.

Table 7. Protective effect of MeOH extract from chungkukjang on TBARS generation in SIN-1-treated LLC-PK_1 cells

Treatment (μg/mL)	MDA (nmole/mg protein)		
	MC	HC	KC
100	0.587±0.011[a]	0.516±0.006[a]	0.507±0.007[a]
250	0.527±0.009[b]	0.491±0.009[b]	0.487±0.009[b]
500	0.332±0.007[c]	0.321±0.006[c]	0.312±0.005[c]
750	0.223±0.006[d]	0.211±0.007[d]	0.204±0.005[d]
1000	0.214±0.006[d]	0.205±0.006[d]	0.191±0.007[e]
SIN-1-treated control	0.824±0.011		
Normal	0.190±0.006		

MC: Moon Chungkukjang, HC: Hyang Chungkukjang, KC: Kim Chungkukjang.
Values are mean±SD.
[a-e]Means with different letters are significantly different ($p<0.05$) from treatment concentration by Duncan's multiple range test.

from 0.824 nmole/mg protein, respectively. The antioxidative effect against $ONOO^-$ of chungkukjang was also confirmed in the present results.

The present results demonstrate the protective effect on free radical-induced oxidative stress. MeOH extracts from chungkukjang significantly preserved the cell viability and inhibited lipid peroxidation in a dose-dependent manner. The result of cell viability and that of lipid peroxidation against NO, O_2^-, and $ONOO^-$-induced oxidative stress are strong in the order of KC>HC>MC. The present study supports the scientific evidence on the antioxidative effect of chungkukjang under cellular oxidative stress model. Furthermore, KC had the strongest antioxidative activity under *in vitro* assay and good score of sensory evaluation, most effectivly protected against NO, O_2^-, and $ONOO^-$-induced oxidative cellular damage.

ACKNOWLEDGEMENTS

This research was financially supported by the Ministry of Commerce, Industry and Energy (MOCIE) and Korea Industrial Technology Foundation (KOTEF) through the Human Resource Training Project for Regional Innovation.

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(Received April 14, 2008; Accepted April 21, 2008)