Antioxidant and antigenotoxic activities of Korean fermented soybean

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Abstract

This study was aimed at evaluating the antioxidative and antigenotoxic activities of Korean fermented soybean (Chungkookjang) in vitro and in vivo. The 100% ethanol extract of Chungkookjang (CKJ) inhibited the generation of 1,1-diphenyl-2-picryl hydrazine (DPPH) radicals, and had an inhibitory effect on LDL oxidation. CKJ and its constituents (genistein and daidzein) also inhibited H2O2-induced DNA damage from NIH/3T3 fibroblasts. Furthermore, they showed the cytoprotective effects against H2O2-induced cell death. In vivo study also demonstrated that an oral administration of CKJ extract (800 mg/kg/day) for 2 weeks potently inhibited the formation of malondialdehyde, the damage of DNA and the formation of micronucleated reticulocytes in KBrO3-treated mice. The well-known antioxidants, trolox and vitamin C, also showed the potent inhibition on these parameters. All these results indicate that CKJ extract may be a useful antigenotoxic antioxidant by scavenging free radicals, inhibiting lipid peroxidation and protecting against oxidative DNA damage. The isoflavones, genistein and daidzein, may contribute to these biological effects of CKJ extract at least in part. Korean fermented soybean (Chungkookjang) is suggested to be a promising functional food which can prevent oxidative stress.

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1. Introduction

Chungkookjang (CKJ) is a fermented soybean product and one of the favorite traditional foods made from Bacillus species in rice straw and boiled soybean in Korea. There have been several reports describing the biological activities of CKJ such as antioxidant, antimicrobial, blood pressure lowering and antidiabetic activities (Kang et al., 1998; Cho et al., 2000; Ryu, 2001; Kim et al., 2003, 2004 Yang et al., 2003). Among its constituents, isoflavones (e.g. genistein, daidzein) are found at high concentrations and they are known to possess the protective effect against oxidative damage related with cancer and atherosclerosis (Barnes et al., 1996; Anthony et al., 1998). Several studies have also shown that some traditional foods containing the fermented soybean consumed in Japan and China such as Natto (Iwai et al., 2002), Miso (Hirota et al., 2000), and Dou-chi (Chen et al., 2005) possessed antioxidant activities. However, the elucidation of antioxidative effect of CKJ is far from complete, despite one previous report demonstrating its antioxidative action (Ryu, 2001). And, it may be important to find antigenotoxic effect of CKJ based on the antioxidative action. Therefore, in the present investigation, CKJ was evaluated for the protective effects against an oxidative stress in vitro and in vivo to clearly establish antigenotoxic and antioxidative activities. For an in vitro study, the effects on the formation of malondialdehyde (MDA) in liver, DNA damage of lymphocytes and the micronucleus formation of peripheral blood against KBrO3-induced oxidative damage were examined. The activities of the isoflavones among the CKJ constituents were also examined.
2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), Dulbecco’s modified Eagles medium (DMEM), phosphate buffered saline (PBS), trypsin-EDTA, and antibiotics were obtained from Gibco BRL (Grand Island, NY). Normal melting point agarose (NMPA), low melting point agarose (LMPA), methyl linoleate, thiobarbituric acid (TBA), Triton X-100, ascorbic acid (Vit-C), genistein, daidzein, and ethidium bromide were purchased from the Sigma Chem. (St. Louis, MO).

2.2. Animals

Specific pathogen-free ICR mice (male, 5 weeks old) obtained from Orient (Seoul, Korea) were used. The animals were given lab chow (Purina Korea) and water ad libitum. They were acclimatized in an animal facility (KNU) under the conditions of 20–22%, 40–60% relative humidity and 12 h/12 h (light/dark) cycle for at least 7 days prior to the experiment.

2.3. Preparation of the ethanol extract of CKJ

Chungkookjang (CKJ) was obtained from Sunchang Food Co. (Sunchang, Korea). CKJ (200 g) was extracted with 10 volumes of 100% ethanol at room temperature for 3 days. After drying under vacuum, approximately 15 g of CKJ ethanol extract was obtained and it was used throughout this study. In order to measure the content of isoflavones, the flavonoid glycosides were hydrolyzed to the corresponding flavonoid aglycones with HCl. The concentrations of genistein and daidzein were determined by HPLC analysis according to the previously published procedures (Setchell et al., 1987). HPLC (Shimadzu 10A, Japan) was performed using an ODS column (150 × 4.6 mm, STR ODS II, Shinwa Chem. Japan). The compounds were eluted with 70% acetonitrile in 0.1% acetic acid at 1.0 ml/min and monitored at 254 nm. The contents of genistein and daidzein were revealed as 1.5 mg/g and 2.0 mg/g CKJ extract, respectively.

2.4. Measurement of antioxidative activity in vitro

The free radical scavenging activity was determined routinely using 60 μM DPPH. The absorbance was measured at 520 nm after incubating the test samples at 37 °C for 30 min according to the previously described procedures (Fugita et al., 1998). For measuring the inhibitory activity of LDL oxidation, LDL (2 mg/ml) was treated with the test samples. LDL oxidation was initiated by adding 0.1 mM copper sulfate to the reaction mixture, followed by incubation at 37 °C for 3 h. The thiobarbituric acid reactive substance (TBARS) was determined following the experimental procedures published previously (Kim and Yang, 2001).

2.5. NIH/3T3 fibroblast culture and single cell gel electrophoresis (comet assay)

NIH/3T3 fibroblasts obtained from the American Type Culture Collection were grown as monolayers in DMEM with 10% FBS, 1% glutamine, and 1% penicillin-streptomycin at 37 °C under 5% CO2. The cytoprotective effect of the CKJ extracts against 5 × 10^{-3} M H2O2 in the NIH/3T3 cell lines were investigated in vitro using the MTT method (Cole, 1986). The cells were plated at 25,000 cells/well in 96 wells. For DNA damage experiment, the cells were plated at 5 × 10^4 cells/well in 24-well plates, and incubated for 30 min with/w/o H2O2 (5 × 10^{-3} M) and the test compounds. Two hours later, the cells were harvested and subjected to single cell gel electrophoresis. To do comet assay, the cells were embedded in agarose on frosted microscopic slides using the previously described procedures (Sing et al., 1988). In brief, 0.65% NMPA in PBS (100 μl) at 65 °C was dropped onto the slides and they were covered with a glass coverslip (18 × 18 mm, No. 1). The cover slip was removed after leaving the slides on ice for 10 min. The cells were mixed with 200 μl of 0.5% LMPA, and 50 μl of the cell suspension was immediately loaded onto the agarose layer on the same slide. The slide was covered with a coverslip and left on ice for 10 min. A final layer of agarose (100 μl of 0.5% LMPA) was applied in the same manner. The slides were immersed in an ice-cold lysis solution (10 mM Tris, pH 10.0, containing 2.5 M NaCl, 100 mM EDTA, 10% DMSO and 1% Triton X-100) at 4 °C for 1 h. The levels of oxidized pyrimidines and purines in DNA were determined by washing the slides twice with an enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 ng/ml BSA, pH 8.0) for 5 min. After incubation for 30 min at 37 °C with formamidopyrimidine-DNA glycosylase (FPG, 1 unit/50 μl) or endonuclease III (Endo III, 1 μg/ml). Electrophoresis was carried out in a tank containing 300 mM NaOH, 1 mM EDTA, pH 13.0 for 15 min under 25 V and 300 mA. The slides were then transferred to 0.4 M Tris buffer solution (pH 7.5), washed three times and gently dried. Ethidium bromide (2 μg/ml) was dropped onto the gel and the slides were examined using a BH2 fluorescence microscope (Olympus, Japan) equipped with a 20×-W2 dichromatic mirror (excitation filter: 515 nm, barrier filter: 590 nm). Image analysis was carried out using the Komet software (version 5.5, Kinetic Imaging, Liverpool, UK) on 50 randomly selected cells. The level of DNA damage was quantified by measuring the increase in the tail length and the olive tail moment (Olive et al., 1990).

2.6. In vivo study

CKJ was dissolved in 0.5% carboxymethyl cellulose (CMC) and orally administered to mice at 800 mg/kg/day for 2 weeks (n = 6). KBrO3 was dissolved in sterile saline and administered to mice at 80 mg/kg/day by intraperitoneal injection at day 11 to day 14 after initial treatment of CKJ. Twenty-four hours after final treatment of KBrO3, blood was withdrawn from cardiac puncture for comet assay as described above, and from tail vein for micronucleus assay. Livers were excised for the following lipid peroxidation experiment. The lipid peroxidation in liver was measured according to the previously described procedures (Ohkawa et al., 1979; Draper and Hadley, 1990). Briefly, each supernatant of homogenized tissue (200 μl) was mixed with 200 μl distilled water, 25 μl BHT, 500 μl 10% phosphotungstic acid in 0.5 N H2SO4, and 0.7% thiobarbituric acid and incubated in boiling water bath for 50 min. After cooling, 1 ml butanol was added and stirred. The mixture was centrifuged and the absorbance of the supernatant was measured at 353 nm. The extent of lipid peroxidation was determined by measuring the quantity of TBARS. 1,1,3,3-Tetraethoxypropane was used as the standard, and the lipid peroxide concentration is expressed as MDA (μg/g/protein). The protein content of homogenate was measured by Lowry method employing bovine serum albumin as standard (Lowry et al., 1951). For micronucleus assay, acridine orange (AO)-coated glass slides were prepared according to the previously reported (Hayashi et al., 1990). The collected blood was placed in a center of AO-coated glass slide and covered with 24 mm × 40 mm coverslip. The AO supravitaly stained reticulocytes (RETS) were examined using fluorescence microscopy with a blue excitation and a yellow barrier filter. The frequencies of the micronucleated reticulocytes (MNRETS) were recorded based on 1000 RETs per mouse.

2.7. Statistical analysis

All the experiments were performed in triplicate. The results were expressed as the arithmetic mean ± S.D. Statistical evaluation of the data was carried out using a Student’s t-test. P-value of less than 0.05 was considered significant.

3. Results

First, the free radical scavenging activities of CKJ extract, its isoflavones and the well-known antioxidants were measured in vitro using DPPH. As shown in Fig. 1a and b, CKJ extract exhibited significant and concentration-dependent radical scavenging activity (14.9–26.6% inhibition) at 150–450 μg/ml, being less active than trolox
and vitamin C. These antioxidants showed strong activity (39.5% and 43.9% inhibition, respectively) even at 7.5 μg/ml. But, the isoflavones, genistein and daidzein, showed very weak inhibition. Against copper-mediated LDL oxidation, CKJ extract possessed the significant inhibitory activity (58.5–67.5% inhibition) at 150–450 μg/ml. The isoflavones, genistein and daidzein, and the antioxidants, trolox and vitamin C, also exhibited the inhibitory activity (30.6%, 27.3%, 51.4%, 39.2% inhibition, respectively) at 7.5 μg/ml as expected (Fig. 2a and b).

When these DNA repair enzymes were treated to H$_2$O$_2$-exposed comet slides, a higher level of DNA damage was observed compared with that of the control group, OTM of 20.3 ± 1.8 and 24.6 ± 1.2, respectively (Fig. 4a). Under these conditions, the isoflavones and the antioxidants exhibited potent inhibitory activity at 2.5 μg/ml against H$_2$O$_2$-induced DNA damage in the presence or absence of the repair enzyme. Under the same conditions, CKJ extract showed the significant and strong inhibitory activity at 50–150 μg/ml (Fig. 4a and b), regardless of the treatment of repair enzymes.

Finally, in vivo effect of CKJ extract was examined on KBrO$_3$-treated mice. As shown in Fig. 5a, CKJ extract possessed the significant inhibitory effect of lipid peroxidation in liver as measured by MDA formation (104.4% inhibition) at 800 mg/kg. Furthermore, CKJ extract inhibited DNA damage assessed by comet assay and apparently reduced MNRET formation of peripheral blood (72.5% and 40.6% reduction) at 800 mg/kg (Fig. 5b and c). As expected, trolox and vitamin C also strongly inhibited these parameters.

4. Discussion

The present study has clearly demonstrated that CKJ extract and its components have significant antioxidative and antigenotoxic activities. Especially, the results of comet analysis suggest that CKJ extract may be a strong antiox-
idative and antigenotoxic agent. The cellular mechanism(s) and the active constituents responsible for the decrease in the level of DNA damage by CKJ extract are not clear. However, it can be speculated that CKJ extract might offer some protection against the DNA strand breakage induced by oxygen free radicals, because comet assay detects DNA breaks in a single cell (Niki and Noguchi, 2000; Festa et al., 2001). And it is possible that the isoflavones including genistein and daidzein may, at least in part, contribute to the protective effects by CKJ. This possibility may be partly supported by the finding that soy isoflavones, genistein and daidzein, had an inhibitory effect on the DNA strand breaks induced by hydrogen peroxide (Forti et al., 2005; Raske et al., 2006). It is significant to note that CKJ extract and its isoflavones also prevented oxidative DNA damage when DNA repair enzymes such as endonuclease III or FPG were used, indicating the protective effects on DNA oxidation (Moller, 2006). CKJ extract also contains considerable amounts of polyphenols (data not shown). These results clearly show the antioxidative activity of CKJ extract, which is due to their components, isoflavons and other polyphenolic compounds.

The most important finding from the present investigation is in vivo antigenotoxic activity of CKJ extract, as demonstrated by inhibition of liver lipid peroxidation, comet analysis and micronucleus assay. Several studies have previously shown the formation of micronuclei induced by KBrO3 both in vitro (Robbiano et al., 1999) and in vivo (Sai et al., 1992). The exposure of KBrO3 in vivo provoked oxidative DNA and chromosomal damage partly through the formation of hydroxyl and NO radicals (Watanabe et al., 2002). CKJ extract greatly reduced the in vivo formation of MDA and MNREts induced by KBrO3 when administered orally. CKJ also exhibited antigenotoxic activity against oxidative DNA damage in vivo, along with well-known antioxidants. All these results clearly indicated that CKJ extract showed antigenotoxic activity probably by its antioxidative action. Therefore, CKJ extract may be a useful natural antigenotoxic antioxidant scavenging free radicals, inhibiting lipid peroxidation and protecting oxidative DNA and chromosomal damage. Further investigation is to be carried out to establish the therapeutic potential to treat several disease conditions that involve oxidative stress.

In conclusion, CKJ extract and its constituents, genistein and daidzein, showed significant antioxidant activity in vitro. CKJ extract exhibited significant free radical scav-
enging effect against DPPH radical generation and had an inhibitory effect on LDL oxidation. CKJ extract also showed significant cytoprotective effects. It strongly inhibited \( \text{H}_2\text{O}_2 \)-induced DNA damage. Furthermore, CKJ extract exhibited \textit{in vivo} antioxidative and antigenotoxic activities. These results indicate that CKJ may be a useful antigenotoxic antioxidant scavenging free radicals, inhibiting lipid peroxidation and protecting against oxidative DNA damage. Further investigations will be needed to evaluate the antioxidative activity of CKJ as well as to characterize the active compounds in detail.

**Conflict of interest statement**

Extracts from Korean fermented soybean (Chungkookjang, CKJ) were evaluated for their protective effects against the generation of free radicals and lipid peroxidation. In addition, the protective effects against \( \text{H}_2\text{O}_2 \)-induced cytotoxicity and oxidative DNA damage in the NIH/3T3 fibroblasts line were examined. Several studies have also demonstrated that traditional foods containing fermented soybean consumed in Japan and China such as Natto, Miso, and Dou-chi have antioxidant activities. However, there are no reports demonstrating the antioxidative activity underlying the antigenotoxicity of CKJ. The ethanol extracts appeared to have most potent antioxidant activities. These results show that the extracts of CKJ may be a useful antigenotoxic antioxidant by scavenging free radicals, inhibiting lipid peroxidation and protecting against oxidative DNA damage without having any cytotoxicity. Therefore, Korean soybean is a promising functional food that can prevent oxidative stress.

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**References**


