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Inhibitory Effect of Korean Fermented Soybean (Chungkookjang) Extract and Genistein Against Trp-P-1 Induced Genotoxicity in HepG2 Cells

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ABSTRACT - This study evaluated the protective effect of Chungkookjang (CKJ) extract, a Korean traditional fermented soybean product made from *Bacillus* species in rice straw and boiled soybean, and one of its main flavonoids, genistein, against Trp-P-1 induced cytotoxicity and DNA damage in HepG2 cells. CKJ and genistein exhibited protective effect against Trp-P-1 induced cytotoxicity and Trp-P-1 induced DNA single strand breaks. CKJ and genistein inhibited Trp-P-1 induced CYP1A1 and CYP1A2 transcription in HepG2 cells. Our results indicated that CKJ and genistein have the protective effect against Trp-P-1 induced cytotoxicity and DNA damage. Via inhibiting expression of CYP1A1 and CYP1A2. CKJ can be used as a promising functional food material that prevents the genotoxicity induced by carcinogens produced by the heat treatment of foods such as heterocyclic amines (HCAs) that cause genomic instability.

Key words: Chungkookjang (Korean fermented soybean), Trp-P-1, HepG2 cells, Cytotoxicity, DNA damage

Heterocyclic amines (HCAs) are created when heating protein foods such as meat and fishes at high temperature. These products are known as substances that have strong mutagenicity and carcinogenicity[1-3]. International Agency for Research on Cancer (IARC) classifies tryptophan-P-1(3-amino-1,4-dimethyl-5H-pyrido [4,3-b] indol, hereinafter referred to as Trp-P-1), which is one of HCAs, into Group 2B (possible human carcinogen)[4].

Trp-P-1 is detected in roasted meats, gravy of heated meats, boiled meats and burned meats. Therefore, there is no way to remove these carcinogens from cooked and processed foods. To avoid being exposed to these carcinogens, it is required to improve cooking methods or eating habits to abstain from foods that contain a lot of them.

Meanwhile, HCAs are converted to substances with strong reactivity that can be combined with DNA by being metabolized by cytochrome P450 1A (CYP1A1/1A2) oxidase in cells[5]. In case of Trp-P-1, nitrenium ions metabolized through N-hydroxylation are combined with DNA and become a substance with genotoxicity including mutation[6].

With the purpose of developing a chemopreventive agent that inhibits carcinogens created while heating and cooking foods, this study was conducted to use Chungkookjang (CKJ), which is one of Korean traditional fermented soybeans, as a genotoxicity inhibitory substance. CKJ that contains a lot of isoflavone polyphenol compounds including genistein is known for major flavonoid having various biological activities including antioxidant activities and estrogenic effect[7]. This study has an objective on investigating the inhibitory effect of Chungkookjang extract and genistein, which is one of the main ingredients of Chungkookjang, against food carcinogens such as Trp-P-1, and their action mechanism.

The result of this study shows that DNA damage caused by Trp-P-1 is inhibited by CKJ extract and genistein and that such inhibitory effect may be related to metabolic enzymes such as CYP1A1 and CYP1A2. This study has an objective to develop CKJ as a chemopreventive agent against food carcinogens taken from heated and cooked foods by using its action mechanism.

Materials & Methods

Material & Reagents

CKJ was obtained from Sunchang Food Co. (Sunchang, Korea). CKJ (1 kg) was extracted with 1 L of 100% ethanol at room temperature for 2 days. After drying under vacuum, the recovery was about 7.5%. The cell line used for this study was HepG2 cells provided by ATCC (HB-8065) and cultured successively in the genotoxicity lab of College of Pharmacy of Kangwon National University according to the purpose of the experiment. For cell culturing, Modified

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eagle medium (MEM) that contains 10% FBS, 2% L-glutamine and 2% Penicillin-streptomycin was used. 3-amino-1,4-dimethyl-5H-pyrido [4,3-b] indol (Trp-P-1) was purchased from Showa Chemical (Okayama, Japan). Cytochrome P450 CYP1A1 isoenzyme, Cyptochrome P450 CYP1A2 isoenzyme, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, resorufin, 7-ethoxyresorufin, 7-methoxyresorufin, potassium phosphate, magnesium chloride, genistein, α-naphtoflavone, β-naphtoflavone, normal melting point agarose, low melting point agarose, Triton X-100, ethidium bromide, (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-tetrazolium (MTT) and Tris were purchased from Sigma-Aldrich (St.Louis, MO). MEM, phosphate-buffered saline (PBS), fetal bovine serum (FBS), trypsine-EDTA, L-glutamine and penicillin-streptomycin were purchased from GIBCO BRL (Grand Island, NY). For all other reagents, guaranteed reagents were used. Total RNA Extraction Kit® was purchased from Bio-Rad, while PCR kit was purchased from Promega. Primer was compounded through Bioneer.

Cytotoxicity Test

To investigate the cytoprotective effect of CKJ extract against the cytotoxicty of Trp-P-1, a microplate reader was used for HepG2 cells according to MTT method[8]. Put 25,000 cells on each well of a 96-well plate and cultured them for 24 hr in 10% FBS-containing batches 90 μl, and then added the sample 10 μl. Cultured them for other 45 min in a CO_2 incubator. After that, Added the MTT reagent 15 μl and then added DMSO 200 μl after 4 hours. After that, measured the absorbance at 570 nm.

Alkaline single cell gel electrophoresis

To evaluate the genotoxicity of Trp-P-1 and anti-genotoxicity of samples, comet assay was applied. Trp-P-1 (final concentration: 5×10^{-4} M in DMSO) was treated alone and together with CKJ extract or genistein simultaneously to evaluate an inhibitory effect respectively. α-naphtoflavone which is an inhibitor, and β-naphtoflavone which is an inducer, were used as a CYP1A1 modulator.

The cells were plated at 1.5×10^6 cells/well in 24 wells, and incubated under 5% CO_2 at 37°C for 24 hr. After that, the cells were treated Trp-P-1 together with (or without) test compound of prescribed concentrations. After 45 min, the media was replaced to new one. The cells were incubated for 1 hr and harvested with trypsin EDTA 500 μl in each well. And the cells were then centrifuged them at 1000rpm for 3 min. The supernatant was removed and subjected to single gel electrophoresis. In brief, the cells slowly were suspended it after adding 0.5% LMPA (low melting point agarose) 200 μl. After taking off the cover of slide with 0.65% NMPA (normal melting point agarose), which was solidified it in a refrigerator, the 50 μl of cell suspension mixed with LMPA was dropped and solidified it in the refrigerator for 30 min after covering the slide again. After taking off the cover again, added 0.5% LMPA 130 μl and solidified it in the refrigerator for 30 min. After that, removed the cover slide, and put it in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, 10% DMSO, 1% Triton X-100), and then dissolved it without light for 60 min. After that, put the slide in electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 20 min without light, and arranged it to the both poles of an electrophoresis apparatus, and then conducted electrophoresis at 25 V and 300 mA for 15 min. After taking out the slide, neutralized it by putting it in 0.4 M Tris (pH 7.5) for 30 min, and dried it in a tray, and dropped ethidium bromide (2 μg/mL) 20 μl to be distributed evenly, and then dyed it after covering it with a cover glass. And the slides were observed with a fluorescence microscope by using a 515-560 nm excitation filter and a 590 nm barrier filter. For the observation, KOMET 5.5 (Kinetic image, England), which is an image analyzer, was used and 50 cells per slide were measured. The comet test data was presented by using olive tail moment (% DNA × distance of center of gravity of DNA, OTM) and tail length (distance between the head and the last DNA fragment, TL)[9-12].

Total RNA isolation and RT-PCR for CYP1A1 and CYP1A2

Trp-P-1 (final concentration: 5×10^{-4} M) was treated after culturing the cells dispensed in a 6-well plate (5×10^6 cells/well) for 24 hours. CKJ extract or genistein were treated by concentration simultaneously with Trp-P-1. After culturing the cells for 5 hr and harvesting them by using trypsin EDTA, and then centrifuging them at 1000rpm for 3 min. After removing the supernatant, RNAs were extracted by using Total RNA Extraction Kit (Bio-Rad) according to the manufacturer's recommendation. After measuring the absorbance at both wavelengths of 260 nm and 280 nm, calculating the RNAs concentration, and then the extracts were stored at −70°C. RT reacted the cDNA at 42°C for 50 min and at 99°C for 5 min by using Gene Cycler Thermal Cycler (Biorad, Gerculis, CA) and compounded them. The applied primer sequences were as follows[13]

β-actin: 5′ CTACAATGAGCTGCTGCGTGTGG 3′
5′ TAGCTCTTCTCCAGGGAGGA 3′
CYP1A1: 5′ TCTTTCTCTTCCTGGCTATC 3′
5′ CTGTCTCTTCCCTTCACTCT 3′
CYP1A2: 5′ GGAGGCCTTCATCCTGGAGA 3′
5′ TCTCCCACTTGGCCAGGACT 3′

For gene amplification, 1 cycle was conducted under β-actin 94°C 3 min condition, and 20-30 cycle were conducted under 94°C 20 sec, 52°C 20 sec and 72°C 40 sec condition. For CYP1A1, 1 cycle was conducted under 94°C 3 min condition, and 20-30 cycles were conducted under 94°C 20 sec, 49°C 20 sec and 72°C 40 sec condition. For CYP1A2, 1 cycle was conducted under 95°C 4 min condition, and 37 cycles were conducted under 94°C 1 min, 60°C 1 min and 72°C 2 min condition. Separated the reaction mixture 8-10 μl by 1.5% agarose gel electrophoresis after the amplification, and confirm the band after dying it with ethidium bromide for 20 min.

Statistical analysis

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

Results

Cytotoxicity inhibitory effect of CKJ extract and genistein

Fig. 1 shows that as a result of the Trp-P-1 cytotoxicity test, CKJ extract and genistein, which is an ingredient of CKJ extract, inhibit Trp-P-1 induced cytotoxicity significantly. The concentrations of CKJ (100 μg/mL) and genistein (10 μg/mL) were determined from preliminary studies. The cell viability was as follows; Trp-P-1 (42.0 ± 1.9), CKJ extract (53.5 ± 2.4), and genistein (62.3 ± 3.1). The negative control was calculated to 100% of cell viability. From the results, CKJ extract and genistein have 11.5% ($p < 0.01$) and 20.3% ($p < 0.01$) of protective effect respectively against 5×10^{-4} M Trp-P-1 induced cytotoxicity. Therefore, it has been suggested that CKJ extract and genistein have protective effect against the cytotoxicity of carcinogen like Trp-P-1.

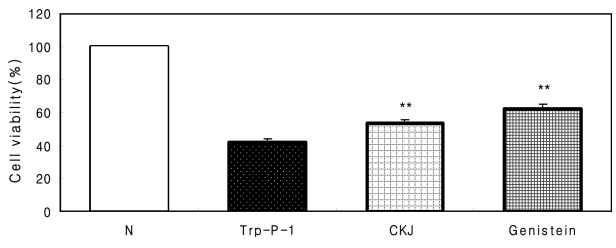

Fig. 1. Cell viability of CKJ extract (100 μg/mL) and genistein (10 μg/mL) toward 5×10^{-4} M Trp-P-1 induced cytotoxicity. These were run with triplicate wells and two independent experiments. N means the solvent control. *$p < 0.05$, **$p < 0.01$, Significantly different from the positive control group (Trp-P-1) by Student's t-test.

DNA damage by Trp-P-1 and inhibitory effect of CKJ extract and genistein

Fig. 2 shows Trp-P-1 induced DNA damage compared by using OTM and TL values. In case of OTM value, DNA damage was as follows; Trp-P-1 (19.8 ± 3.1), CKJ extract (7.9 ± 1.5, $p < 0.01$), genistein (7.3 ± 1.9, $p < 0.01$), α-naphtoflavone (7.3 ± 1.2, $p < 0.01$) and β-naphtoflavone (22.5 ± 2.5) respectively. The negative control was 1.7 ± 0.2. In case of TL value, DNA damage was also as follows; Trp-P-1 (105.9 ± 8.7), CKJ extract (63.8 ± 8.79, $p < 0.01$), genistein (61.5 ± 4.1, $p < 0.01$), α-naphtoflavone (74.1 ± 7.9, $p < 0.01$) and β-naphtoflavone (131.6 ± 13.0, $p < 0.05$) respectively. The negative control was 15.5 ± 1.6. The inhibitory effect of CKJ extract and its ingredient genistein was significant. β-naphtoflavone, which is a metabolism inducer, increased DNA damage, while α-naphtoflavone, which is an inhibitor, reduced DNA damage significantly. The results measured by using OTM and TL value were similar.

Fig. 3 shows the inhibitory effect of CKJ extract between 50, 100, and 200 μg/mL against Trp-P-1 induced damage compared by using OTM and TL value. In case of OTM value, the damage was as follows; Trp-P-1 (24.0 ± 3.2) and CKJ extract 50 μg/mL (8.3 ± 2.5, $p < 0.01$), 100 μg/mL (8.9 ± 2.5, $p < 0.01$) and 200 μg/mL (6.1 ± 1.4, $p < 0.01$). The negative control was 1.2 ± 0.1. In case of TL value, the damage was as follows; Trp-P-1 (115.1 ± 5.6) and CKJ extract 50 μg/mL (69.0 ± 3.3, $p < 0.01$), 100 μg/mL (68.9 ± 8.3, $p < 0.01$) and 200 μg/mL (58.4 ± 6.2, $p < 0.01$), which means that DNA damage was inhibited at 50~200 μg/mL

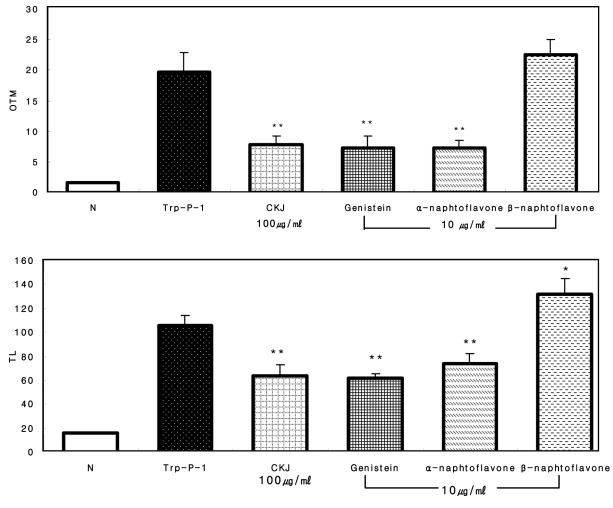

Fig. 2. Inhibitory effect of CKJ extract, genistein, α-naphtoflavone, and β-naphtoflavone against 5×10^{-4} M Trp-P-1 induced DNA damage by comet assay. Olive Tail Moment (OTM), Tail length (TL). Bars represent the average of triplicate determinants and standard deviation. N means the solvent control. *$p < 0.05$, **$p < 0.01$, Significantly different from the positive control group (Trp-P-1) by Student's t-test.

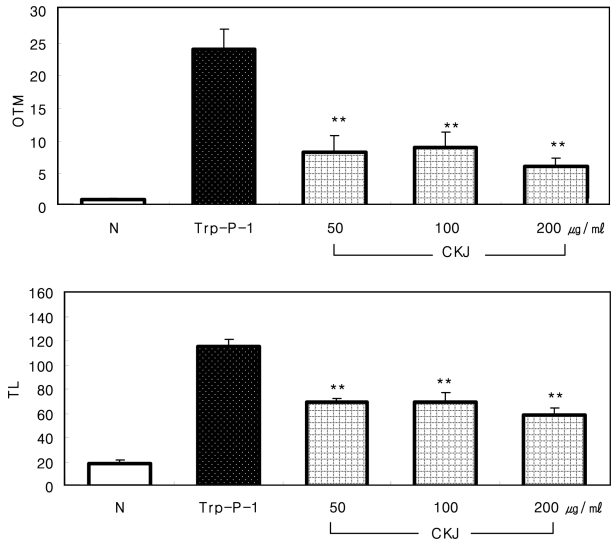

Fig. 3. Inhibitory effect of CKJ extract against 5×10^{-4} M Trp-P-1 induced DNA damage by comet assay. Olive Tail Moment (OTM), Tail length (TL). Bars represent the average of triplicate determinants and standard deviation. N means the solvent control. $*p < 0.05$, $**p < 0.01$, Significantly different from the positive control group (Trp-P-1) by Student's t-test.

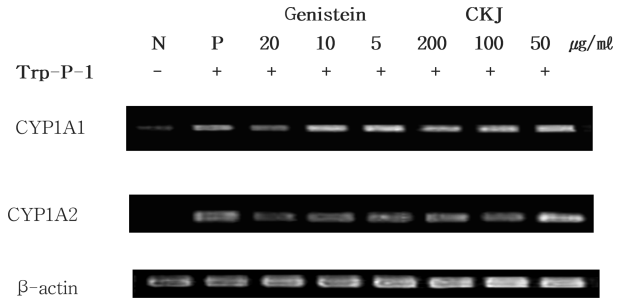

Fig. 4. CYP1A1 and CYP1A2 expression in RT-PCR analysis after being induced by 5×10^{-4} M Trp-P-1 without or with CKJ extract and genistein in HepG2 cells. N means the solvent control. P means Trp-P-1 alone.

tested. The negative control was 18.6 ± 2.8. The results measured by using OTM and TL value were similar in the dose-response study. Therefore, it has been confirmed that CKJ extract and genistein reduce Trp-P-1 induced DNA damage.

RT-PCR of CYP1A1 and CYP1A2

Fig. 4 shows CKJ extract inhibited Trp-P-1 induced CYP1A1 and 1A2 with concentration-dependent manner. The results measured for both CYP 1A1 and 1A2 were similar. Therefore, genistein also inhibited CYP1A1 and CYP1A2 expressions. Genistein 20 μg/mL, which is the maximum concentration, had great inhibitory effect against CYP1A1 and 1A2.

Discussion

HCAs are produced by the pyrolysis of protein, amino acid and creatine when cooking and processing foods at a high temperature[14]. HCAs can be reduced to some degree through the improvement of cooking methods such as temperature down[15,16]. HCAs are N-hydroxylated by CYP1A2 and then metabolized after esterification. Finally nitrenium ions are combined and reacted with the DNA base[17]. A lot of researches on *in vitro* and animal experimental models have reported CYP1A2-induced metabolic activation[18]. CYP1A2-induced metabolic activation is promoted by the intake of HCA-rich foods and also influenced by the polymorphism of phase II enzymes. Cancers caused by HCAs are mainly associated with colorectal cancer and breast cancer. The intake of HCA-rich foods has dynamic association with rectal cancer and causes P53 tumor suppressor gene mutation[19].

HCAs are detected in over 25 foods and divided into 2-amino-3-methyl imidazo(4,5-f)quinoline(IQ)-type HCAs and non-IQ-type HCAs. The former is created when heating the mixture of creatin(in)e, amino acids and sugar. Non-IQ type HCAs are created by the pyrolysis of amino acid and protein at a higher temperature than IQ type HCAs. Roasted meats, gravy of heated meats and boiled meats as well as burned meats contain HCAs. Trp-P-1, which is one of Non-IQ-type HCAs, is converted to a hydroxyl amino derivative by CYP1A2, which is one of cytochrome P450s, and activated by esterification enzymes such as acetyl transferase and sulfotransferase. It creates adducts mainly in the C8 position of guanine and changes the DNA structure by base substitution, deletion or insertion[20].

Recently, researches on modulating the mutagenicity or the carcinogenicity of HCAs have widely reported, and over 180 HCAs including alizarin and zinc protoporphyrin have been discovered[21]. Among them, flavonoid including quercetin has been reported to have inhibitory effect[22-30]. To reduce the carcinogenicity of food-origin carcinogens such as HCAs and benzo(a)pyrene[B(a)P], the followings are discussed. 1) reduce overcooked fish and meat intake 2) improve cooking methods 3) inhibit mutagen formation 4) intercept molecules (absorption) 5) deactivate metabolic activation 6) scavenge electrophiles 7) modulate phase II enzymes and 8) enhance DNA repairs[21]. It has been reported that HCAs are metabolized widely in experimental animals and human bodies and that liver is the main organ for metabolism. It is oxidatively metabolized by CYP1A2, and the main metabolic product is created by the oxidation of heterocyclic ring and ethyl group. Exocyclic amine group CYP1A2-mediated N-oxidation of HCA creates N-hydroxy HCA. Such an oxidative action may occur in extra hepatic tissues by CYP1A1 and CYP1B1[31,32]. Following this, N-

hydroxy-HCA creates unstable ester in liver or extrahepatic tissues by N-acetyl transferase or sulfotransferase, and it creates DNA adduct.

Therefore, CYP1A1 or CYP1A2 will be an important target for the inhibition of the genotoxicity of HCA compounds such as Trp-P-1 through metabolic activation control[21]. There is a report that Indole-3-carbinol contained in cabbage or broccoli inhibits the creation of IQ- and 2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine(PhIP)-induced DNA adducts by inhibiting CYP1A1 and 1A2 as well as the creation of abberant crypt foci (ACF) in rats[33-36]. There is another report that epigallo-catechin-3-gallate, which is tea polyphenol, inhibits human CYP1A2-mediated activation by PhIP[37]. Like this, there are a lot of reports on the inhibitory effect of natural substances against the genotoxicity of food carcinogens. CKJ and its ingredient, which are the samples of this study, also perform inhibitory activities of CYP1A1/1A2.

As a Korean tranditional food, CKJ has been recently reported by scientific researches to perform various biological activities. CKJ is known for antioxidative, antimicrobial, blood pressure dropping and anti-diabetic effects as well as for isoflavones (e.g. genistein and daidzein), which are antioxidative ingredients[38-45].

We have reported that CKJ extract showed potent anti-genotoxicity against B(a)P induced DNA damage through the inhibition of CYP1A1/1A2 expressions and their activities[46] This study has confirmed that CKJ extract and genistein have protective effect against Trp-P-1 induced cytotoxicity. And the strong inhibitory effect of CKJ extract and genistein against Trp-P-1 induced DNA damage has been confirmed. CKJ extract 50, 100 and 200 μg/mL resulted in the significant reduction of DNA damage with concentration-dependent manner. When CKJ extract and genistein were treated with Trp-P-1 simultaneously, they inhibited CYP1A1 and CYP1A2 at mRNA level. All the result shows that the inhibitory effect of genistein is better than CKJ extract under experimental conditions.

There is a report that apigenin and chrysin inhibit the creation of B(a)P-induced DNA strand breaks and adducts by reducing CYP1A1, and quercetin inhibits it by reducing CYP1A2[47]. There is another report that quercetin inhibits the creation of B(a)P induced DNA adducts[48] and metabolic activation[49] by inhibiting CYP1A1. Flavonoids such as flavones and flavonols inhibited Trp-P-2 induced DNA damage, and the result of Trp-P-2 induced mutagenicity in Ames test shows their antimutagenic effect[50] through the inhibition of CYP1A1. Genistein and daidzein inhibited the CYP1A1 metabolism of B(a)P in mouse hepatoma cells[51] and the genotoxicity in human mammary ephithelial cells by controlling the glutathione S-transferase system[52]. Genistein and daidzein inhibited the activation of 2,3,7,8-tetrachloro dibenzodioxin (TCDD) induced CYP1A1 and the covalent bond between CYP1A1 mediated DNA and B(a)P[51].

It has been reported that as a result of Ames assay on Korean fermented soybeans such as CKJ extract and its ingredients such as genistein and genistin, they have inhibitory effect against substances such as aflatoxin B1 and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced mutagenicity[53]. It has been also reported that as a result of Ames assay, genistein and daidzein inhibit Trp-P-1 induced SOS induction (umu gene expression) and mutation in Ames test[54].

Antioxidative agents such as butyl hydroxy toluene (BHT) and propyl gallate inhibit the creation of HCAs and the genotoxicity induced by HACs[55]. We cannot rule out the possibility that CKJ extract and genistein which is one of its isoflavone compounds may have inhibitory effect against the genotoxicity caused by antioxidant activation including the scavenging activity of free radicals created during the polyphenolic component-induced metabolic process as well as against CYP1A1 and CYP1A2 metabolic enzymes[56].

It is considered that CKJ or its extract that prevents the carcinogenic actions of food carcinogens coming into human bodies inevitably when taking heat treated protein foods can be used as preventive substances for cancers by improving the genome instability of human bodies. Further investigations will be needed to evaluate the action mechanism of CKJ and isoflavones against HCA induced mutagenicity.

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국문요약

청국장추출물과 청국장의 주요한 플라보노이드의 하나인 genistein의 HepG2 세포에서 Trp-P-1 유도 세포독성과 DNA손상에 대한 보호효과를 평가하였다. 청국장추출물과 주요 플라보노이드성분 genistein은 Trp-P-1 유도 세포독성에 대하여 세포독성보호효과를 나타내었다. 청국장추출물은 Trp-P-1 유도 DNA single strand breaks를 억제하였다. 한편, 청국장추출물은 HepG2 세포에서 Trp-P-1 유도에 의한 CYP1A1와 CYP1A2 발현의 억제를 나타내었다. 청국장추출물과 genistein은 Trp-P-1에 의한 유도 세포독성과 DNA손상에 대하여 CYP1A1, CYP1A2 발현억제에 의하여 보호효과가 나타나는 것으로 판단된다. 한국의 전통 콩발효식품인 청국장은 게놈 불안정성(genomic instability)을 일으키는 heterocyclic amines (HCAs)과 같은 식품의 가열조리로부터 올 수 있는 발암물질에 대한 유전독성을 예방

할 수 있는 유망한 기능성물질로서 활용가능성이 있을 것으로 판단된다.

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