Yonsei Medical Journal
Vol. 29, No. 2, 1988

Mutagenic and Anti-Mutagenic Properties of Meju and Other Korean Food Products from Fermented Soybeans

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In order to investigate the mutagenic activity of Meju, an important component of the Korean diet, both chemical techniques and the Ames test were used. To determine if antimutagenic activity is present in Meju and other soybean based foods, the Ames Test was done in the presence of aflatoxin B_1, benzo(a)pyrene, and other mutagens. Although aflatoxin contamination was found in 6 of 43 samples of Meju tested, the amounts were less than 1 ppb in all but one. Meju had a protective effect against mutations produced by both aflatoxins and benzo(a)pyrene, both of which act via an epoxide, but not against other mutagens tested.

Key Words: Antimutagenic activity. meju, aflatoxin B_1, benzo(a)pyrene. ames test.

As hepatocellular carcinoma is very common in Southeast Asia (Waterhouse 1982), a strategy to decrease the incidence of this condition could save many lives. The association of liver cell cancer with chronic hepatitis B infection has been demonstrated in other parts of the world (Harris 1984), and is probably operative in Korea also, where the incidence of hepatitis B virus carrier status is between 10 and 15% (Chung 1986). A role for aflatoxin as a co-carcinogen in the origin of hepatocellular carcinoma has also been proposed (Harris 1984). This study was initiated in order to investigate the possible influence of Korean fermented foods in this process, as several food products in daily use, such as soy sauce and bean paste, originate from a common source. Termed Meju, this source is a soy bean paste loaf traditionally fermented by various bacterial and fungal species.

Investigators have shown that certain *Aspergillus* species which produce aflatoxins are often present in Meju; Lee Su-Rae (1985) has reviewed efforts to find such mycotoxin contamination of the Korean food supply. Kim and Roh (1985) did a survey of soy bean food products, using a high pressure liquid chromatography (HPLC) assay for aflatoxins. Their results suggested that earlier assays for aflatoxins with less sophisticated techniques had given erroneously positive results, as they found no detectable aflatoxins in 70 samples of Meju. The present study was initiated in order to further investigate the possibility that such fermented food products contain aflatoxins or other carcinogenic substances.

The use of the "Ames Test", a *Salmonella typhimurium*/mammalian microsome mutagenicity assay (Ames *et al.* 1975, Maron & Ames 1983) is widely accepted as a predictor of the carcinogenicity of many substances, including mycotoxins (Ueno 1978). In order to test for such substances, this test was utilized, in conjunction with HPLC and other standard chemical methods, to investigate Meju and other foods produced from it.

However, there is also much interest recently in substances that can have a protective effect carcinogens (Miller *et al.* 1978, Ames 1986) particularly substances which are present in common foods. Epidemiological studies by Hirayama (1982 & 1986) and others have shown that the incidence of many different cancers, including that of the liver, varies with life styles, particularly with the intake of green and yellow vegetables, which Hirayama defines as those

Received December 15, 1987
Accepted February 26, 1988
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This study was supported by the Joint Research Association of Yonsei University Medical College and Christian Medical Research Center at Presbyterian Medical Center.
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containing more than 600μg carotene per 100g. During the course of these studies, it was found that the Meju preparations being investigated appeared to have such a protective effect against mutagens, rather than being mutagenic. Therefore, further studies were done to investigate this, as the possibility that the incidence of hepatocarcinoma can be reduced through dietary change is an exciting one.

METHODS

Sample Collection

Forty-three Meju samples prepared in the winters of 1985 and 1986 were collected from rural households in Chonbuk, Chonnam and Kyungnam Provinces, in south central Korea. For aflatoxin assays and mutagenicity tests individual samples were used. A homogeneous composite sample, prepared from six different aflatoxin-free Meju samples from the three provinces, from both years, was used for most of the antimutagenicity tests; this preparation is termed "composite Meju". One sample each of Doenjang (soy paste) and Kanjang (soy sauce) from Chonbuk Province was also used for mutagenicity and antimutagenicity tests.

Sample Preparation

All samples were extracted using the procedure of DeVries and Chang (1982) modified as follows: use of a blender (3×30 sec), centrifugation (2500× g, 10min), and use of the whole filtrate to increase senstivity. Samples were further purified on a Waters silica Sep Pak column previously activated with 5cc chloroform. The column was washed with 2cc hexane, then 2cc ether, and eluted with 5cc chloroform: methanol (9:1). After concentration, samples were applied to thin layer chromatography (TLC) plates. Analtech 20×20cm plates (#10511) were used, with samples dissolved in benzene: acetonitrile (98:2) and a mobile phase of chloroform: acetone (88:12), containing varying amounts of water to compensate for varying humidity.

Fractions were scraped off the plate, eluted with acetone: chloroform (3:7), evaporated under nitrogen, and redissolved in acetonitrile for assay by high performance liquid chromatography (HPLC). Aflatoxins B_1, B_2, G_1 and G_2 were determined for each sample.

Derivatization with Trifluoroacetic Acid (TFA)

To improve the sensitivity of the HPLC assay for aflatoxins B_1 and G_1, TFA derivatives were made. The procedure of Cohen and LaPointe (1981) was modified to use smaller volumes, and the final sample was dissolved in acetonitrile and filtered through a 0.45 micron filter.

High Performance Liquid Chromatography

Non-derivatized and TFA-derivatized samples were assayed for the presence of aflatoxins using a Waters HPLC system consisting of two 510 pumps, a U6K injector, a uBondapak C18 column (3.9mm×30cm), a 680 gradient controller, and a 740 data module. The detector was a Perkin Elmer LS4 Fluorescence Spectrometer set at excitation 368nm, emission 452nm. The solvent system consisted of water: acetonitrile: acetic acid (180:820:10), 90% and acetonitrile 10%, at 1.2 ml/min for non-derivatized samples. For TFA derivatized samples, the percentage of acetonitrile was decreased to 6%. The sensitivity of this system is 4pg for aflatoxin B_1 and 10pg for B_2, G_1 and G_1 (AFB1, AFB2, AFG1 and AFG2).

Confirming the identity of a putative aflatoxin peak was made by comparing the retention times of putative peaks with those of standard peaks and by the spiking of standard to samples. Next, the same confirming methods were used with a second HPLC solvent system (water: methanol 1:1). If this also indicated the presence of aflatoxin, fluorescence wavelengths were changed to determine if the putative peak increased or decreased to the same extent as an authentic aflatoxin standard. Samples were reported positive for aflatoxin only when both confirmatory tests were satisfied.

Aflatoxin standards (Sigma Chemical, St. Louis, MO, USA) were dissolved in methanol, stored at 0°, and used as standards for TLC and HPLC. Only Meju samples containing no detectible aflatoxins were used to screen for carcinogenic activity.

Mutagenicity and Anti-Mutagenicity Tests

Samples were prepared for mutagenicity and antimutagenicity testing by the same extraction and silica column chromatography method used for samples before aflatoxin analysis. A few samples were separated by TLC and the individual fractions tested. Solvents used in the extraction procedure were distilled before use because of antimutagenic activity in solvent residues. Because the antimutagenicity activity was not completely removed by distillation, a "solvent blank" was prepared for each experiment, starting with water but no sample, following the whole

prcoedure, and dissolving the residue in dimethylsulfoxide to test for antimutagenic activity. For each result reported, the effect of the solvent has been subtracted. The solvent inhibitory activity correction varied from 0 to 15.1%. All glassware used in this portion of the work was cleaned with chromic acid solution also, to remove any detergent or other residue with antimutagenic activity. For samples that were fractionated by TLC, plates containing no binder were used, as antimutagenic activity was extracted from plates containing binder. Each sample was tested for mutagenicity and anti-mutagenicity activity in the *Salmonella typhimurim*/mammalian microsome bioassay of Ames (Ames *et al.* 1975, Maron & Ames 1983). The work was done with strain TA 98 of *Salmonella*. For mutagenicity tests, food extracts were added in dimethylsulfoxide by the standard method. For antimutagenicity tests, appropriate amounts of mutagen (AFB1, benzo(a)pyrene, 2-aminofluorene or 4-nitroquinoline) were added with the food extract. Controls included solvent blanks as well as mutagen alone, and, for negative control, dimethylsulfoxide, the vehicle used to dissolve food extracts and mutagens. Inhibition of mutations was calculated from revertant colonies produced by sample in the presence of mutagen, less negative control, and revertant colonies from mutagen alone, less negative control.

Preincubation experiments were done by the method of San and Chan (1987), with modifications detailed in Table 4.

For toxicity tests, representative samples of food extracts and solvent residuces were tested with both strains of *Salmonella typhimurium*, in the presence of the mammalian microsome fraction used in the mutagenicity test (Felton 1981). Toxicity of all samples tested was much lower than corresponding antimutagenicity activity, ranging from 0 to 12%. The toxicity of the "solvent blank" ranged from 1.3 to 8.3%; thus, some of the antimutagenic activity of solvent residues can be attributed to toxicity. Reported results are the average of duplicate or triplicate determinations.

RESULTS

Aflatoxin Screening

Forty three samples of Meju were assayed for aflatoxins, thirty three 1985 and two 1986 samples from Chonbuk Province, and eight 1986 samples from Chonnam and Kyungnam Provinces. The results from the six samples, all from Chonbuk, in which detectable amounts of aflatoxins were found, are presented in Table 1.

Table 1. Aflatoxin content of Meju samples which were confirmed to contain the aflatoxin

Sample Number	concentration (ppb)			
	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
1	0.3	ND*	0.5	0.1
2	ND	ND	0.8	ND
3	ND	ND	0.33	0.15
4	ND	ND	4.8	ND
5	0.09	0.16	0.08	0.06
6	ND	ND	ND	0.08

* ND: non detected

Mutagenicity Tests

Representative samples of Meju were tested from mutagenicity, from all three provinces represented. Fractions further purified by TLC were also tested for a few samples. Various amounts of Meju were extracted, so that the amount of extract added to a plate varied from an equivalent of 0.65gr initial Meju weight to that equivalent to 20gr. The number of mutations seen in all these experiments was no different from those seen with the negative control, containing dimethylsulfoxide only. One sample each of Doenjang (soy paste) and Kanjang (soy sauce) from Chonbuk were also tested for mutagenicity. These foodstuffs are both produced from Meju; neither contained any mutagenic activity.

Anti-Mutagenicity Tests

Meju samples were tested for anti-mutagenic activity, in the presence of 0.1μg AFB1, 10μg 2-aminofluorene and 1 μg benzo(a)pyrene, all of which require activation by mammalian microsomes, and in the presence of 1μg 4-nitroquinoline, which does not require activation. One sample each of Doenjang and Kanjang was also tested for antimutagenic activity with the mutagens. Results appear in Table 2. All compounds tested were found to inhibit the mutagenicity of AFB1 and benzo(a)pyrene; there was no effect on 2-aminofluorene or 4-nitroquinoline mutagenicity.Various fractions of individual Meju samples purified by TLC were also tested with ABF1; the inhibition of

Table 2. Inhibition of mutagenicity by the organic extracts from Meju and its food products

Mutagen	Sample for antimutagenic effect test*	enzyme activation (S9)	No of net* revertant colonies	inhibition rate (%)
Aflatoxin	blank	+	280	–
B_1 0.1μg	3.4 gr Doenjang	+	159	43.4
	3.4 gr Kanjang	+	208	25.7
	3.4 gr Meju	+	21	92.5
	0.2 gr Meju	+	67	76.1
	0.05 gr Meju	+	182	35.1
	0.01 gr Meju	+	205	26.6
Benzo(a)pyrene	blank	+	715	–
1.0 μg	3.4 gr Meju	+	53	92.6
2-Aminofluoren	blank	+	373	–
10 μg	3.4 gr Meju	+	372	0
4-Nitroquinoline	blank	–	820	–
1 μg	3.4 gr Meju	–	818	0

* net revertant colony = revertant colony – spontaneous revertant colony

Table 3. Antimutagenic activity of Meju fractions from TLC in the presence of AFB1

Band	Rf Value	% Inhibition of AFB1 Mutations
1 (origin)	0	88
2	0.05-0.07	3
3	0.15-0.16	21
4	0.19-0.2	58
5	0.31-0.33	62
6 (Rf of aflatoxins)	0.5 -0.93	6

A 1986 Meju sample from Chonnam Province was extracted by standard procedures, and chromatographed on TLC. Eluates from 6 TLC areas were tested for antimutagenic activity, with strain TA 98 and 0.1 μg AFB1. Mutagen alone resulted in 314 revertant colonies; negative control, 20.

Table 4. Effect of preincubation on antimutagenic activity of Meju extract

Exp. No.	Preincubation	Final Incubation	Percent Inhibition
1.	–	–	0
2.	Meju	–	100
3.	–	Meju	43
4.	solvent	–	41
5.	–	solvent	40

5×10^8 cells strain TA 98 were preincubated 20 min at 37°C with 0.1 μg AFB1 + microsome fraction + component in column 2, centrifuged, washed with phosphate-buffered saline and mixed with top agar + component in column 2, centrifuged, washed with phosphate-buffered saline and mixed with top agar + component in column 3 for final 48 hr incubation. Meju = extract from 0.5 gr Meju. Solvent = solvent blank equivalent to that for 0.5 gr Meju extract. Dimethylsulfoxide concentration = 5% in preincubation, 1% in final incubation.
negative control = 14 revertant colonies

mutations by various fractions varied from 3 to 88%. A representative experiment is shown in Table 3. The highest anti-mutagenic activity is seen in the fraction containing the components left at the origin, while the lowest amount of activity is seen in the area directly above the origin, and in the area corresponding to the retention time of aflatoxin standards.

In experiments done with 10μg AFB1+strain TA 98 but no microsome fraction, mutation frequency was no higher than the negative control (data not shown). When Meju extracts and solvent blanks were tested in preincubation experiments, inhibitory activity was seen only when Meju extract was present during the 20 minute preincubation with AFB1, microsome fraction and bacteria (Table 4). When the extract was added later, after the bacteria were spun down, and washed with phosphate-buffered saline, the colony count was no different from that seen with solvent blank alone.

DISCUSSION

Although 14% of the Meju samples were found to contain detectable amounts of aflatoxins, concentrations were very small, with only one sample containing over 1ppb. US and World Health Organization allowable limits for these mycotoxins are 15 and 30ppb respectively, while Korea has set no allowable limits. Concentrations of AFG1 and AFG2 higher than those of AFB1 have been reported only once previously, in a recent survey of foods from Swaziland (Peers *et al.* 1987).

The Meju preparations studied contained components that have a protective effect against some mutagens. Similar effects of Meju were found by Lee (1985), who fed rats N-metyl-N'-nitro-N-nitrosoguanidine plus Meju, which lowered the incidence of gastroduodenal sarcoma significantly, compared with rats fed the gastric carcinogen alone. Epidemiological studies by Hirayama (1982) on the effect of soy bean paste soup, which is made from a Japanese soy bean peparation similar to Meju, showed that daily consumption of this product reduced standard mortality rates for gastric cancer.

Because the Meju extract exhibited the antimutagenic activity only when present with bacteria plus AFB1 plus microsome fraction, it is likely that it is the activation step that is affected by the active component(s). There are several possible mechanisms for this effect. AFB1 is a procarcinogen, and must be activated to an epoxide to interact with the genome (Stark 1986). The details of this enzymatic activation by the Phase I microsomal enzymes are nuclear. Other pathways for detoxification lead to AFQ1, AFM1 and other products (Moss & Neal 1985). These metabolites, as well as the epoxide, are subject to conjugation by Phase II enzymes, leading to sulfates, glucuronides, and glutathione conjugates. Thus, a protective effect can stem from an interaction with the aflatoxin molecule, or with the epoxide produced, or from interference with the activation pathway. Or it can be secondary to an effect on an enzyme pathway which alters the balance of metabolites or conjugates produced. Interference with binding to DNA is another possible mechanism, as the reaction of the epoxide is very quick, and probably takes place during the 20 min preincubation.

It has been shown that AFB1-mediated mutagenesis is decreased by a number of different compounds. San and Chan (1987) investigated six phenolics with such activity, finding that all six reduced the mutations from 30μM AFB1 in a dose dependent manner; this is a toxic dose, and a factor of 270 times the amount used in our study. Their investigation of resulting metabolites suggested that aflatoxin metabolism was inhibited by the phenols, but there was no direct interaction with AFB1. Other workers have shown various effects of phenolic compounds on microsomal enzyme systems, with results ranging from enhancement to inhibition of mutagenesis by various mutagens (reviewed in San and Chan 1987).

Bhattacharya *et al.* (1987) tested the effects of 19 vitamins on AFB1 in the mammalian bioassay system, and found retinoids, riboflavin, folic acid, menadione, cyanocobalamin, ascorbic acid and pyridoxine to be significantly antimutagenic. Because of the kinetic data suggesting that retinol and menadione interact with some components(s) of the microsomal enzyme system, the authors postulate those vitamins may be acting as substrates for the monooxygenase, thus interfering with the reaction necessary for AFB1 activation.

Kensler *et al.* (1987) investigated a series of 5-membered cyclic sulfur-containing compounds (1,2-dithiol-3-thiones) present in cruciferous vegetables, and showed AFB1-DNA adduct levels were decreased in the livers of rats fed such compounds during exposure to AFB1 by gavage. Focal areas of hepatocellular alteration were quantitated to evaluate the effects of these compounds on preneoplastic lesions in the rats; results indicated a decrease in such presumptive pre-cancerous areas when the sulfur-containing compounds were included in the diet. This indicates that an effect on AFB1 metabolism can also be seen in the mammalian cell, although this effect may be mediated by alterations in glutathione levels or other effects on conjugation pathways that are not present in the bacterial cell.

Benzo(a)pyrene is also a procarcinogen, activated via formation of an epoxide (Selkirk 1978). Wood has found that plant phenols and riboflavin-5'-phosphate (FMN) inhibit mutagenicity of benzo(a)pyrene in the Ames Test, by reacting directly with the epoxide to catalyze its hydrolysis (Wood 1982a and 1982b). Buening (1981) showed that naturally occurring flavonoids affect liver metabolism of benzo(a)pyrene by affecting the activity of monooxygenase enzymes.

As the extracts tested contain so many components, and the effects seen with plant products on mutagenicity are very complex, it is difficult to speculate what actual compound(s) are involved in this effect. However, as mutagenicity of AFB1 and benzo(a)pyrene, but not of 2-aminofluorene or 4-nitroquinoline, was decreased, components which

hydrolyze the epoxide or interfere with the microsomal monooxygenase that produces the epoxide are probably present. The experiment with TLC fractions of Meju emphasizes that there are several components responsible for the effect seen with the unfractionated preparation. It is very likely that soybeans contain many of the same compounds investigated by others, as outlined above. The final extract which was used for the mutagenicity experiments undoubtedly includes mot of the hydrophobic molecules, such as flavonoids and retionl.

It is dangerous to extrapolate results from bacteria to mammalian systems. Many compounds mutagenic to *Salmonella* have also been shown to be carcinogenic, but there has been no work done to correlate antimutagenic properties with anticarcinogenesis. For any antimutagenic component in a food product to have a protective effect against a liver carcinogen, the component must be present in the liver, where the carcinogen is activated and has its effect on DNA. It is not possible to determine if the antimutagenic components of Meju are present in the liver until they are identified.

Also, the properties of the microsomal metabolizing system for aflatoxins vary considerably from species to species, and the carcinogenicity of aflatoxins also vary, but not always in a way that can be readily explained from the properties of the enzyme systems involved (Stark 1986). The activities of detoxification systems, such as metabolic pathways that produce readily excretable compounds rather than epoxide, and the glutathione conjugating system which eliminates the epoxide, are all very important in determining the ultimate carcinogenicity of AFB1 in any particular species or individual. For these reasons, experiments using rat microsomal activation systems may not be relevant to humans. However, our results suggest that there may be components in these fermented food products that inhibit the activity of other, cancerproducing components, and could serve as a protective agent against such substances.

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