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Assurance on the Genotoxicological Safety of Fermented Vegetables Pasteurized by Gamma Irradiation

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Abstract The genotoxicological safety of fermented vegetables pasteurized by gamma irradiation was examined to consider the possibility of the application of irradiation for extending of fermented vegetables. A fermented vegetable was irradiated at 20 kGy to assure its toxicological safety even at a high dose of radiation. The Ames test with *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537) and *Escherchia coli* (WP2), and the chromosomal aberration test in Chinese hamster lung (CHL) cells were performed. *In vivo* micronucleus test were conducted in mouse bone marrow cells. With or without metabolic activation, negative results were obtained in the Ames test and the chromosomal aberration test. In the micronucleus test, there was no enhancement in the formation of micronucleus, and there were no such significant differences between the irradiated and non-irradiated samples. The observed results indicated that, a level of 20 kGy of gamma irradiation on the fermented vegetable did not bring about any genotoxic effects under the described experimental conditions.

Keywords: gamma irradiation, *Kimchi*, genotoxicity, Ames test

Introduction

Gamma irradiation has become an effective pasteurization method and is utilized widely for pasteurization of foodstuffs, agricultural and hygienic products. It has been confirmed that gamma irradiation inactivates the pathogenic or saprogenic microorganisms present in the foodstuffs, without bringing about any major changes in the original quality of food. Recently, it has been reported that gamma irradiation has significant positive effects on the regulation of the aging, improvement of the hygienic quality, and the elongation of the shelf life of traditional Korean fermented foodstuffs (1-4). These studies reported that the nutritional, physiological, physicochemical, and sensory characteristics of fermented foods were stable within up to a dose of 10 kGy of gamma irradiation. However, studies on the safety of gamma irradiated fermented foods, which is the most important consideration for the consumer's acceptance and industrial application, are limited. World Health Organization (WHO) concluded that irradiation with an average dose of 10 kGy of gamma radiation does not bring about any toxicological, and specific nutritional or microbiological problems in food commodity. (5). Many other studies have also confirmed that food irradiation process is the most clean and safe pasteurization method for food preservation (6). In spite of these conclusions, many consumers and managers working in the food industries require a safety insurance against each irradiated item, especially for fermented vegetables, as their safety has yet not been specified.

Genetic toxicological studies play a central role in development of new chemicals for pharmaceutical and agricultural use. Genotoxicity tests are considered as a short-term process in nature, and they are an integral part of a product's safety assessment. Usually, a set of four assays constitutes the minimum test battery that satisfies the global requirements. The set includes a bacterial reverse mutation assay, *in vitro* cytogenetic test with a mammalian cell culture, *in vitro* gene mutation assay in mammalian cell cultures, and *in vivo* rodent bone marrow micronucleus test (7, 8).

The objective of this study is to confirm the toxicological safety of fermented vegetables (*Kimchi*) pasteurized by a high dose (20 kGy) of gamma irradiation. The test system used in this study allowed the direct evaluation of the gamma irradiated samples of *S. typhimurium, E. coli,* Chinese hamster lung cells (*in vitro* test) and mouse bone marrow cells (*in vivo* test).

Materials and Methods

Preparation of the fermented vegetables The Chinese cabbage was cut into half and dipped in a 15% salt solution for 4 hours. Each piece of cabbage was washed with tap water, drained, and then mixed with spices and additives. The preparation of the spice mixture and fermentation process was performed according to the method of Song *et al.* (2).

Gamma irradiation Approximately 300 g of fermented cabbage was packed in polyethylene vinyl bags and irradiated in a cobalt-60 gamma irradiator (point source, AECL, IR-79, Nordion, Canada) at the dose of 20 kGy. The source strength was approximately 100 kCi with a dose rate of 70 Gy min^{-1} at 15±0.5°C and the actual doses

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were within ±2% of the target dose. Dosimetry was performed using 5-mm-diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany). A non-irradiated control was also prepared. The irradiated and non-irradiated fermented vegetables (50 g each) were extracted separately with distilled water (500 mL) and methanol (500 mL) at room temperature for 24 hr. The extracts were filtered through Whatmann No. 2 filter paper, concentrated in a rotary evaporator, and dried under vacuum.

Ames test The Ames *Salmonella*/microsome mutagenicity test was performed according to the method of Maron and Ames (9). Strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535, TA 1537), and *Escherchia coli* (WP2) were used in the present study. The strains were kindly provided by the Korea Research Institute of Chemical Technology (Daejeon, Korea). Strains were tested for their genetic traits such as the histidine or tryptophan requirements, deep rough (*rfa*) characteristic, UV sensitivity (*uvr*A or B mutation), and ampicillin resistant by the R-factor before they were used. For metabolic activation, S-9 mix, containing 10% of the S-9 fraction (purchased from Oriental Yeast Co., Ltd., Tokyo, Japan) was used. The 10% of S-9 solution was prepared from the livers of Sprague-Dawley rats. The test samples were dissolved in water and tested at five dose levels. Triplicate plates were run and the results were shown as the mean±S.D. The test sample was considered to be mutagenic when the number of counted colonies exceeded the number of colonies in the negative controls by at least a double value. The relationship between the dosage level and response was observed.

***In vitro* chromosomal aberration test** *In vitro* chromosomal aberration test was assayed as per the methods of Ishidate *et al.* (10), and Dean and Danford (11) with some modifications. The Chinese hamster lung cell was chosen to detect the chromosomal aberrations (12), and was supplied by the Korea Research Institute of Chemical Technology (Daejeon, Korea). The test samples were dissolved in a medium, and tested at three dose levels. The test was carried out in the absence and presence of the metabolic activation S-9 mix. A total of 200 metaphase spreads (100 per duplicate flask) were examined and scored for the chromatid-type and chromosome-type aberrations. Gaps were scored (data not shown), but were not included in the percentage of the cells with the chromosomal aberrations. The frequency of the polyploid metaphase cells was determined, based on the scoring of 100 metaphase cells per culture.

Mouse bone marrow micronucleus test The study protocol was based on the method of Heddle *et al.* (13) and was in compliance with the corresponding OECD guideline (14). Male ICR mice (7-weeks old, average body weight 30 g) were obtained from the Korea Research Institute of Chemical

Table 1. Ames test with *S. typhimurium* (TA 98, TA 100, TA 1535, TA 1537) and *E. coli* (WP2) strain in the presence of a water-soluble fraction of gamma irradiated fermented vegetable

Test Sample	S-9 mix	Dose (μg/plate)	Number of revertant colonies per plate (mean±S.D.)				
			TA98	TA100	TA1535	TA1537	WP2 *uvr*A
H_2O	−		59±2	130±19	17±2	19±4	25±5
	+		66±5	131±17	20±3	27±2	24±6
Non-irradiated	−	10000	75±7	156±11	25±9	23±1	31±4
	−	5000	71±8	144±1	23±10	22±2	27±2
	−	2500	64±7	135±4	19±9	22±1	26±1
	−	1250	63±8	134±6	21±10	21±2	25±4
	−	625	61±9	125±5	18±2	18±1	21±1
	+	10000	88±4	158±11	26±1	25±4	32±1
	+	5000	81±1	155±7	24±1	22±6	30±1
	+	2500	841±1	160±5	25±3	28±1	23±2
	+	1250	78±1	138±5	18±2	26±5	29±9
	+	625	69±2	119±8	23±6	24±1	29±4
20kGy-irradiated	−	10000	68±3	171±11	29±4	26±1	30±1
	−	5000	59±5	164±13	25±1	23±1	27±2
	−	2500	58±4	163±1	25±6	21±1	22±1
	−	1250	56±1	171±10	19±6	21±1	23±0
	−	625	46±10	159±20	20±5	16±1	21±5
	+	10000	82±1	179±1	29±0	33±1	26±1
	+	5000	66±8	179±8	25±2	29±2	21±1
	+	2500	67±9	157±5	23±4	26±4	24±0
	+	1250	62±6	149±2	19±9	22±5	26±1
	+	625	63±9	139±6	22±1	23±4	25±1
Positive control	−		506±69[a]	772±94[b]	52±48[b]	231±27[c]	177±24[a]
	+		873±39[d]	1408±82[d]	456±13[e]	782±25[e]	403±74[e]

Positive control agents: [a]4-Nitroquinoline-1-oxide (0.5 μg/plate), [b]Sodium azide (0.5 μg/plate), [c]9-Aminoacridine (50 μg/plate), [d]2-Aminoanthracene (0.4 μg/plate), [e]2-Aminoanthracene (2 μg/plate).

Technology (Daejeon, Korea). Five mice were used for each treatment condition. The test samples were dissolved in distilled water, and were administered by intraperitoneal (i.p.) injection in a constant volume of 10 mL/kg body weight. The number of micronucleated polychromatic erythrocytes was counted for every 2,000 polychromatic erythrocytes and the ratio of the polychromatic and normochromatic erythrocytes was noted down.

Statistical analysis Genotoxic activities were expressed as induction factors, i.e. as multiples of the background levels. Analysis of the variance (one-way ANOVA) of the repeated measurements was carried out for the experiments using SAS software (15). Dunnett's test was used to compare each treatment group with the control group. A P value of less than 0.05 was considered as statistically significant.

Results and Discussion

Ames test The Ames test is based on the set of *Salmonella typhimurium* strains that are reverted to histidine independence upon exposure to mutagens (9, 16). The test becomes more sensitive when a tryptophan-dependent *Escherichia coli* strain is included in the study (17). In the present study, TA98, TA100, TA1535, and TA1537 strains of *S. typhimurium*, and WP2 strain of *E. coli* were used. None of the bacterial strains showed an increase in the revertants in the presence of the irradiated fermented vegetable extracts, with or without metabolic activation (Table 1, 2). Also, there was no dose-dependent increase in the number of revertants for the different extract concentrations, and there wasn't any reduction in the bacterial background lawn. Dose levels tested in the assay were selected on the basis of results on the dose range finding study conducted for a tester strain TA100 of *S. typhimurium*, both in the presence and absence of S-9. Seven concentrations of test samples were tested in triplicate up to concentrations of 5,000 μg/plate, and there wasn't any increase in the number of revertants. Therefore, the highest concentration of the fermented vegetable extracts used in the subsequent mutation assay was 10,000 μg/plate. At least two independent assays were performed with triplicate plating for each test sample, and the results from both the trails showed negative result. The negative and strain-specific positive control values were within our laboratory background historical control data range, indicating that the test conditions were adequate and the metabolic activation system functioned properly. Inclusion of a concurrent positive control in every experiment as a means of quality control is recommended, to ensure that the assay is performed according to the prescribed standards and the samples are analyzed accurately. Based on the results of this study, it is concluded that the irradiated fermented vegetable did not bring about any mutagenic or

Table 2. Ames test with *S. typhimurium* (TA 98, TA 100, TA 1535, TA 1537) and *E. coli* (WP2) strain in the presence of methanol-soluble fraction of gamma irradiated fermented vegetable

Test Sample	S-9 mix	Dose (μg/plate)	Number of revertant colonies per plate (mean±S.D.)				
			TA98	TA100	TA1535	TA1537	WP2 *uvr*A
H_2O	−		59±2	130±19	17±2	19±4	25±5
	+		66±5	131±17	20±3	27±2	24±6
Non-irradiated	−	10000	63±4	157±8	23±1	24±6	29±4
	−	5000	63±11	152±13	26±8	26±5	24±1
	−	2500	54±4	141±7	24±2	20±5	22±6
	−	1250	58±3	134±20	19±0	21±6	23±7
	−	625	60±4	130±5	16±3	17±2	25±4
	+	10000	94±4	165±9	29±0	32±1	28±3
	+	5000	74±5	158±11	25±8	29±1	23±3
	+	2500	69±6	158±8	27±3	29±3	25±4
	+	1250	71±1	147±4	18±4	25±3	19±0
	+	625	66±4	141±13	21±5	27±3	22±5
20 kGy-irradiated	−	10000	68±4	179±8	28±0	30±6	32±3
	−	5000	61±5	172±10	23±3	28±2	26±1
	−	2500	66±2	166±14	25±8	30±3	26±4
	−	1250	59±1	144±13	17±1	27±2	22±3
	−	625	61±2	142±14	20±1	23±3	18±4
	+	10000	93±6	177±3	28±8	37±2	27±1
	+	5000	60±4	176±6	29±5	39±1	23±7
	+	2500	68±3	170±4	24±5	33±4	21±5
	+	1250	66±11	165±16	20±3	31±1	22±3
	+	625	62±3	148±6	20±2	32±1	23±4
Positive control	−		506±69[a]	772±94[b]	524±8[b]	231±27[c]	177±24[a]
	+		1873±39[d]	1408±82[d]	456±13[e]	782±25[e]	403±74[e]

Positive control agents: [a]4-Nitroquinoline-1-oxide (0.5 μg/plate), [b]Sodium azide (0.5 μg/plate), [c]9-Aminoacridine (50 μg/plate), [d]2-Aminoanthracene (0.4 μg/plate), [e]2-Aminoanthracene (2 μg/plate).

Table 3. Chromosomal aberration test in Chinese hamster lung cell in the presence of water-soluble fraction of gamma irradiated fermented vegetable

Test Sample	Treatment			Abnomalities (mean)					Aberrant Meta Phases (mean)	Total Aberrations (mean)
	Dose (μg/mL)	Time (hours)	S-9 mix	CsB	CsE	CtB	CtE	PP+ER		
Control		6	−	0	0	0	0	0+0	0	0
		24	−	0	0	0.5	0	0.5+0	0.5	0.5
		6	+	0	0	0	0	0+0	0	0
Non-irradiated	5000	6	−	0	0	1	0	1.5+0	1	1
	2500	6	−	0	0	1	0	2+0	1	1
	1250	6	−	0	0	0	0	0+0	0	0
	5000	24	−	1	0	1	0	0.5+0	2	2
	2500	24	−	1	0	0	0	1+0	1	1
	1250	24	−	0	0	1	0	0.5+0	1	1
	5000	6	+	1	0	0	0	1.5+0	1	1
	2500	6	+	0	0	1	0	1+0	1	1
	1250	6	+	0	0	1	0	0.5+0	1	1
20kGy-irradiated	5000	6	−	0	0	0	1	1+0	1	1
	2500	6	−	1	0	0	0	0.5+0	1	1
	1250	6	−	0	0	0.5	0	1+0	0.5	0.5
	5000	24	−	1	0	0	0	0.5+0	1	1
	2500	24	−	0	0	1	1	0+0.5	2	2
	1250	24	−	0	0	1	0	0.5+0	1	1
	5000	6	+	1	0	1	0	1.5+0	2	2
	2500	6	+	1	0	1	0	1+0	2	2
	1250	6	+	0	0	2	0	0.5+0	2	2
EMS	400	6	−	5	0.5	2	30	2+0	22[a]	37.5[a]
	300	24	−	4	1	6	24	1.5+0	20[a]	35[a]
CPA	50	6	+	28	1.5	15	90	1+0	68.5[a]	134.5[a]

CsB; Chromosome break, CsE; Chromosome exchange, CtB; Chromatid break, CtE; Chromatid exchange, PP; Polyploid, ER; Endoreduplication, EMS; Ethylmethanesulfonate, CPA; Cyclophosphamide.
[a]Significantly different from the control with $P<0.01$.

promutagenic effects under the described Ames test conditions.

***In vitro* chromosomal aberration test** Chromosomal aberrations (CA) are one of the important biological consequences which occur on human exposure to ionizing and other genotoxic agents. Many types of cancers are associated with specific types of CA (18). The CA test measures the ability of toxicant/mutagen/genotoxicant to cause chromosomal aberration in cell cultures. The test samples were assayed for the induction of chromosomal aberration in Chinese hamster lung cell up to the maximum concentration of 5,000 μg/mL in the absence and presence of the S-9 mix, recommended by OECD guideline (19).

Irradiated fermented vegetable extracts were dissolved in a medium and, tested at three dose levels with the highest dosage of 1,250 to 5,000 μg/mL. No statistically significant increase in the structural chromosome aberrations (brakes or exchanges of chromatid and chromosome) was observed in the activated or non-activated study at any of the dose levels tested. And also no statistically significant increase in the numerical aberrations was observed in the study at any of the dose levels tested with or without S-9 activation (Table 3, 4). Appropriate negative control cultures were included in the test system and had low incidences of chromosomal aberrations within the historical solvent control range. Ethylmethanesulfonate and cyclophosphamide were used as positive control chemicals in the absence and presence of liver S-9, respectively. Both compounds induced statistically significant increases in the incidence of chromosomal aberrations ($P<0.01$). Treatment of the cells with the test sample both in the absence and presence of S-9 resulted in a number of aberrations and they were similar to those observed in the concurrent negative controls. Therefore, it was concluded that the irradiated fermented vegetable was unable to induce chromosomal aberrations in the Chinese hamster lung cell when tested up to 5,000 μg/mL, either in the absence or presence of S-9.

Mouse bone marrow micronucleus test The rodent bone marrow micronucleus test is currently the most commonly performed assay for detecting the genotoxic potential of test items *in vivo*. The test is based on the observation that, in many mammalian cells, the chromosome fragments lag in anaphase and are not included in the daughter nuclei that are formed during telophase. Instead, these fragments become membrane bound and form micronuclei. Fragments of chromatin may occur due to chromosome breakage or spindle dysfunction (20). In the present study, a single dose of 2,000 mg/kg of the test sample as an upper limit

Table 4. Chromosomal aberration test in Chinese hamster lung cell in the presence of methanol-soluble fraction of gamma irradiated fermented vegetable

Test Sample	Treatment			Abnomalities (mean)					Aberrant Meta Phases (mean)	Total Aberrations (mean)
	Dose (μg/mL)	Time (hours)	S-9 mix	CsB	CsE	CtB	CtE	PP+ER		
Control		6	–	0	0	0	0	0+0	0	0
		24	–	0	0	0.5	0	0.5+0	0.5	0.5
		6	+	0	0	0	0	0+0	0	0
Non-irradiated	5000	6	–	0	0	0	1	1+0	1	1
	2500	6	–	0	0	0.5	0	1.5+0	0.5	0.5
	1250	6	–	0	0	0	0	0.5+0	0	0
	5000	24	–	0	0	0.5	0.5	1+0	1	1
	2500	24	–	0	0	1	0	0.5+0	1	1
	1250	24	–	0	0	0.5	0	0+0	0.5	0.5
	5000	6	+	1	0	0.5	0	2+0	1.5	1.5
	2500	6	+	1	0	0	0	1+0	1	1
	1250	6	+	0	0	0.5	0	1.5+0	0.5	0.5
20kGy-irradiated	5000	6	–	1	0	1	0	1+0	2	2
	2500	6	–	0	0	0.5	0	1+0	0.5	0.5
	1250	6	–	0	0	0	0	0+0	0	0
	5000	24	–	1	0	0	1	1.5+0	2	2
	2500	24	–	1	0	0.5	0	1+0	1.5	1.5
	1250	24	–	0	0	0.5	0	1+0	0.5	0.5
	5000	6	+	1	0	0	1	2+0	2	2
	2500	6	+	0	0	0.5	0	1.5+0	0.5	0.5
	1250	6	+	0	0	1	0	1+0	1	1
EMS	400	6	–	5	0.5	2	30	2+0	22[a]	37.5[a]
	300	24	–	4	1	6	24	1.5+0	20[a]	35[a]
CPA	50	6	+	28	1.5	15	90	1+0	68.5[a]	134.5[a]

CsB; Chromosome break, CsE; Chromosome exchange, CtB; Chromatid break, CtE; Chromatid exchange, PP; Polyploid, ER; Endoreduplication, EMS; Ethylmethanesulfonate, CPA; Cyclophosphamide.
[a]Significantly different from the control with P<0.01.

Table 5. The rodent Bone marrow micronucleus test in the presence of gamma irradiated fermented vegetable

Irradiation	Test Sample	Dose (mg/kg)	No. of Animals	MNPCE/2000PCE (mean±S.D.)	PCE/NCE+PCE (mean±S.D.)
Non-irradiated	Water		6	0.67±0.82	0.46±0.02
	Water-soluble fraction	2000	6	1.00±0.89	0.45±0.03
	Methanol-soluble fraction	2000	6	1.33±1.03	0.45±0.02
20 kGy-irradiated	Water-soluble fraction	2000	6	1.17±1.17	0.46±0.01
	Methanol-soluble fraction	2000	6	1.50±1.05	0.45±0.01
	Cyclophosphamide	30	6	27.17±1.87[a]	0.44±0.02

[a]Significantly different from the control with $P<0.01$.

was chosen for the genotoxicity test in correspondence to the OECD guidelines (14). At this dose level (the maximum feasible dose), male mice exhibited no toxic reactions (Table 5). The frequency of MNPCEs did not showed a statistically significant increase at 2,000 mg/kg treatment level when compared with the concurrent vehicle control. In contrast, orally administered CPA (positive control) at a level of 30 mg/kg showed a significant increase in the frequency of micronuclei formation ($P<0.01$).

According to Schmid (21), the ratio of polychromatic to mature erythrocytes is fairly constant, with a proportion of 1:1; and cytotoxic compounds have been reported to affect this proportion (21, 22). Cytotoxic effects on bone marrow cell proliferation were evaluated by scoring the population of PCE and NCE, and by calculating the percentage of PCE according to the formula of PCE/PCE+NCE. The proportions of PCEs to NCEs are given in Table 5. There were no significant differences between the test sample (gamma irradiated fermented vegetable extract) injected group and the water injected control group for the PCE/NCE ratios, indicating the absence of bone marrow toxicity in the presence of gamma irradiated fermented vegetable.

In conclusion, the results of the present series of genetic toxicity studies indicate that gamma irradiated fermented

vegetables are not genotoxic. In all the *in vitro* assays performed with the exogenous activation system, there was no apprecaible effect of the S-9 enzymes on activation. This indicates that an irradiated fermented vegetable does not contain compounds susceptible for activation into genotoxic or mutagenic metabolites. Additionally, no evidence of the *in vivo* clastogenic potential was shown in the screening study for mice. These results correlate well with the data reported in our laboratory using Korean soybean fermentation foods with irradiation treatment (23). Furthermore, the WHO (5) reported that a large number of toxicological studies, including carcinogenicity bioassays and multigeneration reproductive toxicology evaluations, have not demonstrated any short-term or long-term toxicity of the food stuffs pasteurized by irradiation process. Therefore, gamma irradiation at 20 kGy or below is evaluated as an effective technique to extend the shelf life of fermented vegetables without any genotoxicity.

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