Journal of Food and Drug Analysis

Volume 20 | Issue 4 Article 14

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Journal of Food and Drug Analysis, Vol. 20, No. 4, 2012, Pages 832-838 doi:10.6227/jfda.2012200412

Antitumor Cell Proliferation and Antigenotoxic Effects of Sufu and Koji Granule, the Chinese Traditional Fermented Product of Soybeans

JING-YI CHEN[1], YING-JANG LAI[2], SHU-HUA CHIANG[3] AND CHENG-CHUN CHOU[1]*

[1.] *Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan, R.O.C.*
[2.] *Department of Food Science, National Quemoy University, Kinmen, Taiwan, R.O.C.*
[3.] *Department of Food and Beverage Management, Taiwan Hospitality & Tourism College, Hualian, Taiwan, R.O.C.*

(Received: March 5, 2012; Accepted: July 18, 2012)

ABSTRACT

Sufu is traditional Chinese fermented product of soybean. In the present study, sufu was manufactured by ripening salted tofu cubes in *Aspergillus oryzae*-fermented rice-soybean koji mash at 37°C for a period of 16 days. The methanol extracts of the non-fermented tofu cubes, sufu, and koji granules collected at various ripening periods were examined for their antiproliferative effect against human colon cancer cells, Caco-2 and HT-29, as well as their suppression effect against the genotoxicity of 4-nitroquinoline-N-oxide (4-NQO) on Intestine-470 cells. It was generally found that the extracts of tofu cubes and koji granules exhibited an antiproliferative effect against the cancer cells in a dose-dependent manner. However, greater antiproliferative activity was noted with the extracts of the ripened sufu and koji granules. Moreover, the antiproliferative effect against cancer cells increased during the ripening period. The investigation of the suppression effect against genotoxicity revealed that the extracts of tofu cubes and koji granules before ripening showed no protective effect on Int-470 against the genotoxicity of 4-NQO, yet the extracts of the ripened sufu and koji granules did exhibit antigenotoxicity against 4-NQO. This antigenotoxicity increased during the ripening period.

Key words: sufu, koji granule, ripening, antiproliferative effect**,** antitgenotoxic effect, 4-nitroquinoline-N-oxide

INTRODUCTION

Epidemiological studies have shown that the consumption of soy-based food that contains antimutagenic factors such as isoflavones, trypsin inhibitors, saponin and phytic acid, might lead to the lower rates of breast, prostate, and colon cancer in Asian countries compared to other parts of the world[(1-3)]. Based on these studies, an avoidance of exposure to mutagens and a diet rich in nutritious foods containing antimutagens have been suggested as an appropriate strategy to reduce the rate of mutation and thus the incidence of cancer in humans[(4,5)].

Sufu is a highly flavored, soft cheese-like product made from tofu cubes of soybean. This fermented soybean product has been produced in China for more than a thousand years. It is usually consumed as an appetizer or a side dish with breakfast rice or steamed bread in China and has an estimated annual production of over 300,000 tons[(6)]. There are various methods used to produce sufu[(6)]. However, enzyme-ripened sufu and mold-fermented sufu are the most common types available in the market. These two types of sufu, the koji enzyme-ripened sufu and mold-fermented sufu, use different fermentation processes and starter organisms. The former requires a shorter fermentation period and is generally less salty and sweeter than the latter[(6,7)]. Considering the lack of scientific data on enzyme-ripened sufu, a series of studies concerning the biochemical, physical, and functional properties of the koji enzyme-ripend sufu have been conducted in our laboratory[(7-11)]. We have found that the koji-ripened sufu contained an enhanced content of aglycone, the bioactive form of isoflavone[(9)]. Besides, the koji-ripened sufu, was also found to possess increased antioxidant and antimutagenic properties as a result of the ripening process[(10,11)]. In the present study, the methanol extracts of sufu and koji granules obtained across various ripening periods were further examined for their effects on both the proliferation of human colon cancer cells, Caco 2 and HT-29, as well as the genotoxicity of 4-nitroquinoline-N-oxide (4-NQO) on Int-407 cells.

* Author for correspondence. Tel: 886-2-3366-4111;
Fax: 886-2-2362-0849; E-mail: fstcchou@ntu.edu.tw

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MATERIALS AND METHODS

I. *Preparation and Ripening of Sufu*

In the present study, sufu was prepared with the enzyme ripening process as described by Li *et al*.[7] Briefly, tofu cubes (ca 2.5 × 2.5 × 2 cm in size) were first mixed with salt and water at a ratio of 4 : 1 : 3 (w/w/w) and kept at room temperature for 24 h. Then they were air-dried for 24 h to obtain salted tofu cubes. Simultaneously, *A. oryzae* was grown in a mixture of steamed rice and soybean at 35°C, with a relative humidity of 70% for 40 h. The prepared koji was mixed with syrup (65% sucrose) at a ratio of 1 : 5 (w/w) and stored at room temperature for 48 h to prepare the rice-soybean koji mash. The sufu was then ripened by mixing the salted tofu cubes with the koji mash (175 g) in a glass jar, where the ratio of koji granules: syrup was 1 : 4 (w/w) and held at 37°C for a period of 16 days.

II. *Sampling and the Preparation of Methanol Extract*

During the sufu ripening period, bottles containing sufu and koji granules were withdrawn at specific intervals. Sufu and koji granules were separated, dried by a freeze-dryer (Free Dry System/Freezone®4.5, Labconco, MO, USA), homogenized, and kept at -20°C until analysis.

To prepare the methanol extracts, the dried powder of tofu cubes, sufu or koji granules were mixed with methanol (1 : 10, w/v), and held at ca 25°C for 24 h with gentle (120 rpm) shaking. After filtering through Whatman No. 1 filter paper, the methanol extracts were vacuum concentrated and freeze-dried.

III. *Cell Cultures*

In the present study, human Int-407 (ATCC CCL-6) cells were obtained from Prof. Gow-Chin Yen (National Chung Hsing University, Taichug, Taiwan). Human colon cancer cell lines including HT-29 (ATCC HTB-38) and Caco-2 (ATCC HTB-37) were provided by Prof. Tzu-Ming Pan (Institute of Microbiology and Biochemistry, National Taiwan University, Taipei, Taiwan).

Int-407 cells were grown in Basal Medium Eagle (BME, Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with 10% calf serum (Hyclone Laboratories, Inc.), 100 units penicillin/mL, and 100 μg streptomycin/mL (Hyclone Laboratories, Inc.). Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc), 100 units penicillin/mL, and 100 μg streptomycin/mL. HT-29 cells were grown in RPMI 1640 medium (Hyclone Laboratories, Inc) supplemented with 10% fetal bovine serum (Gibco BRL), 100 units penicillin/mL, and 100 μg streptomycin/mL (Hyclone Laboratories, Inc.). Both colon cancer cells were incubated at 37°C in a humidified incubator (NU-4950 DH Autoflow CO_2 Air-Jacketed Incubator, NuAire Inc., Plymouth, MN, USA) of 95% air and 5% CO_2. After trypsin detachment, cell density was determined using hemocytometer.

IV. *Study on Antiproliferative Effects on Human Colon Cancer Cells*

The MTT-based colorimetric assay as described by He *et al*.[12] and Ewaschuk *et al*.[13] was followed to quantify the antiproliferative effects of fermented and non-fermented black soybean extracts. HT-29 or Caco-2 cells in 100 μL culture medium at a concentration of 1×10^5/mL cells per well were placed in a 96-well microplate. After incubation at 37°C in 5% CO_2 in air for 24 h, an aliquot of 20 μL phosphate buffered saline (PBS, pH 7.4) with or without test sample, and 180 μL culture medium, were added to each well on the plates. The HT-29 or Caco-2 cells were washed with PBS twice after 24 h. Then 200 μL of serum-free culture medium that contained 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) was added to each well. After another 4 h of incubation to allow the formation of MTT formazan, DMSO (200 μL. Merck, Darmstadt, Germany) was then added to dissolve the blue crystals and the absorbance was measured at 570 nm (VersaMax™ Tunable Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). The antiproliferative effects were evaluated by comparing the proliferation of treated cell lines to that of the control (PBS without test sample). The viability (%) was calculated according the following formula. Proliferation (%) = [OD(sample)/OD(control)] × 100%. OD (sample) and OD (control) represent the absorbance of the test samples and the control, respectively.

V. *Assessment of Genotoxicity*

To assess the antigenotoxic effect of the methanol extracted test samples, Intestine 407 cell line was seeded in a 96 well plate (1×10^4 cells/well), The medium was removed after 12 h of incubation. Then 200 μL BME medium containing 20 μL test sample (methanol extract of tofu, sufu or koji granule) and 20 μL 4-NQO at a final concentration of 500 μg/mL and 0.2 μg/mL, respectively, was added. 4-NQO was replaced with PBS as the normal control, and PBS instead of the test sample was used as the positive control (4-NQO alone). The methanol extract at the dosage level examined was found to show no cytotoxicity on Int-407 (data not shown). After 12 h of incubation, the medium was removed and 0.05% trypsin-EDTA (50 μL) was added to the cells. Cells were kept at 37°C for 1 min to detach cells. An equal amount of the medium was added to quench trypsin-EDTA. Intestine 407 cells were then centrifuged for 5 min at 285 ×g at 4°C.

To examine the protective effect of the test samples, the extent of the DNA damage induced by 4-NQO was then monitored by the comet assay as described by Singh *et al*.[14] and Ou *et al*.[15] DNA migration (damage) was analyzed by fluorescence microscopy (Nikon Instruments Inc., Melville,

NY, USA). For the evaluation of DNA migration, 50 randomly selected cells were scored for each sample. As shown in Figure 1, cells were categorized as Class 0 (undamaged DNA, no tail), Class 1 (minimal migration, tail length less than a quarter of the head diameter), Class 2 (medium migration, tail length between a quarter and a full of head diameter), Class 3 (extensive DNA damage, tail length greater than the head diameter) and Class 4 (extensive DNA damage, comet with poorly defined or small head).

VI. *Determination of Dried Weight*

The Association of Official Analytical Chemists (AOAC)[16] method was used to determine the dried weight.

VII. *Statistical Analysis*

Mean values and standard deviations were calculated from the data obtained from three separate experiments. Means were analyzed using unpaired two-tailed Student's *t*-test and compared using Duncan's multiple range test in SAS, version 8 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

I. *Viabilities of Caco-2 and HT-29 Cells in the Presence of Sufu and Koji Granule Extracts*

Table 1 shows the viability of the Caco-2 and HT-29 cells treated with the methanol extract obtained from the non-fermented tofu and sufu across various ripening periods. It was generally noted that extracts of the non-fermented tofu and sufu, regardless of ripening period, exhibited an inhibitory effect on the proliferation of the tumor cells examined. The antitumor cell proliferation effect increased as the dosage level of the extract was increased. For example, the Caco-2 and HT-29 cells showed a proliferation of 96.97 and 92.36% in the presence of 1 μg/mL non-fermented tofu extract, while the proliferation reduced to 91.84 and 75.76% as the dosage level of non-fermented tofu extract increased to 500 μg/mL. The extract of sufu, regardless of ripening period, exhibited a significantly higher ($p < 0.05$) antitumor cell proliferation effect as compared to that of the non-fermented tofu cubes since the proliferation of Caco-2 or HT-29 cells was significantly lower ($p < 0.05$) in presence of sufu extract than that in presence of the non-fermented tofu extract. Furthermore, it was found that the antitumor cell effect exerted by the sufu

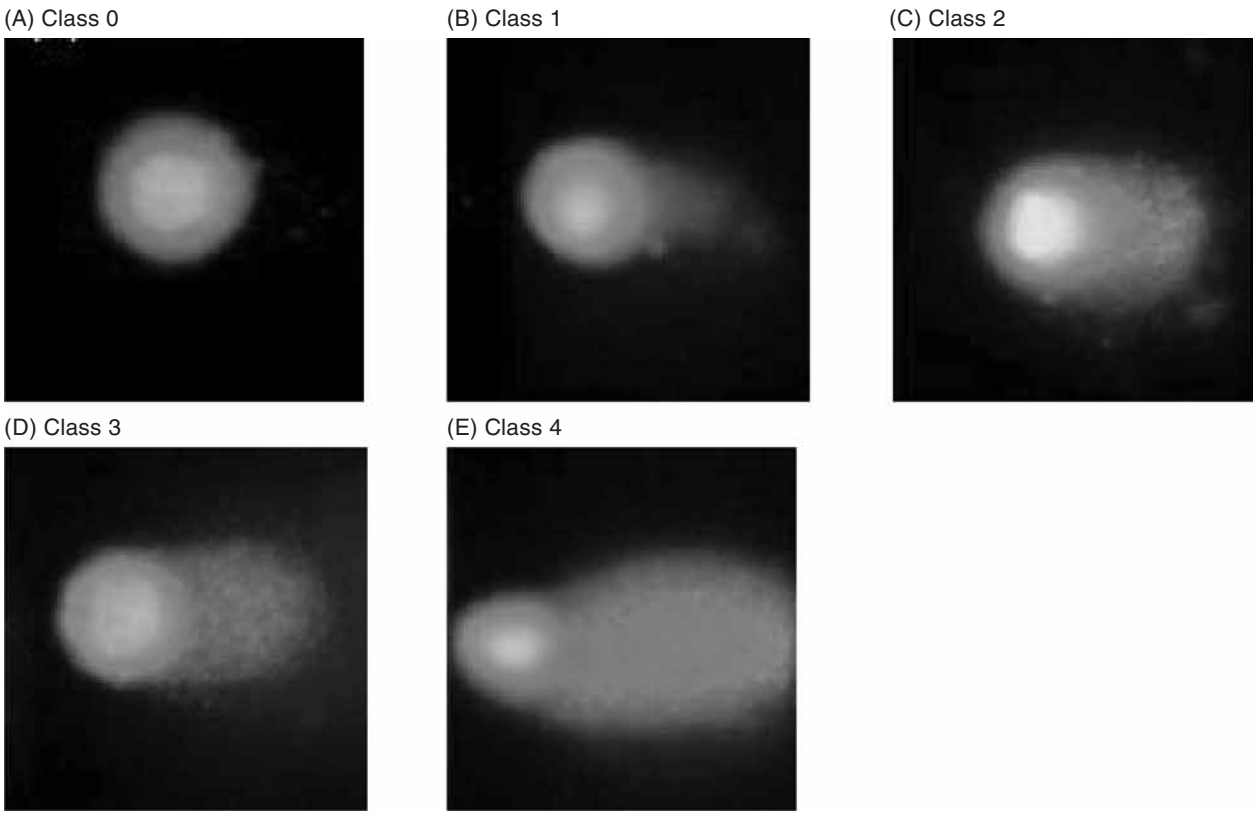

Figure 1. Photomicrographs with various of DNA damage grades in comet assay. Int-407 cells were treated with test sample. (A) Class 0, no tail; (B) Class 1, comets with tiny tail tail length less than a quarter of head diameter; (C) Class 2, comets with medium tail tail length between a quarter and a full of head diameter; (D) Class 3, comets with long tail tail length greater than head diameter; (E) Class 4, comets with poorly defined or small head.

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Table 1. Proliferation of colon cancer cells treated with methanol extracts of sufu

Sample (μg/mL)	Proliferation (%)[a]					
	1	5	25	125	250	500
……..…… Caco-2 cell…….....						
Control[b]	A100.00 ± 4.30a[c]	A100.00 ± 2.35a	A100.00 ± 1.39a	A100.00 ± 3.58a	A100.00 ± 3.99a	A100.00 ± 1.58a
Non-fermented tofu	A96.97 ± 1.61a	A96.26 ± 1.11a	A96.13 ± 2.96ab	B93.82 ± 0.54ab	B93.80 ± 0.27b	B91.84 ± 2.49b
2 h-sufu	BC91.68 ± 2.87a	C89.73 ± 2.42ab	C87.98 ± 2.29ab	C87.86 ± 1.11ab	C87.15 ± 1.80b	C85.88 ± 2.82b
8 day-sufu	C85.57 ± 0.87a	D83.09 ± 0.86b	D79.75 ± 0.95c	D79.50 ± 0.19c	D79.45 ± 1.02c	D72.09 ± 2.66d
16 day-sufu	D80.44 ± 2.21a	E77.61 ± 2.79ab	E75.24 ± 1.20bc	E74.60 ± 2.32bc	E72.54 ± 1.41c	E63.52 ± 1.07d
……..…….HT-29 cell…….....						
Control	A100.00 ± 1.68a	A100.00 ± 1.76a	A100.00 ± 1.33 a	A100.00 ± 1.15 a	A100.00 ± 0.75 a	A100.00 ± 4.73 a
Non-fermented tofu	A92.36 ± 0.17a	B89.63 ± 4.54ab	B88.25 ± 2.99 ab	B87.96 ± 3.28 ab	B85.23 ± 1.65 b	B75.76 ± 1.10 c
2 h-sufu	BC83.69 ± 1.12a	CD81.70 ± 4.68ab	C78.22 ± 1.50 b	C73.84 ± 2.51 c	CD71.24 ± 2.28 c	C69.33 ± 1.50 c
8 day-sufu	CD78.77 ± 0.04a	D78.34 ± 2.39a	D74.37 ± 1.16 ab	C72.55 ± 2.32 b	CD67.96 ± 3.95 c	D64.33 ± 3.21 c
16 day-sufu	D72.74 ± 0.25a	E65.95 ± 2.75b	E65.93 ± 0.62 b	D65.87 ± 3.18 b	D64.67 ± 1.89 b	D62.14 ± 2.62 b

[a]Proliferation was determined after 24 h of incubation and was expressed as percent of the control.
[b]Control is treated with PBS.
[c]Each value represents mean ± SD (n = 3). Means with different letters in the same row (a, b, c) and in the same column for the same cell line (A, B, C, D, E) were significantly different by Duncan's multiple range test ($p < 0.05$).

extract increased as the ripening period was extended. Among the various sufu extracts examined, the extract of the 16 day-sufu showed the highest inhibitory effect on the proliferation of Caco-2 and HT-29 cells since at the same dosage level, the proliferation of cancer cells in this treatment was the lowest among the various sufu extracts examined. For example, the proliferation of the Caco-2 cells and HT-29 cells was only 63.52 and 62.14% in presence of 500 μg/mL of the 16 day-sufu extract. While at the same dosage level, a higher proliferation of 72.09-85.88 and 64.33-69.33%, respectively, was noted for Caco-2 cells and HT-29 cell in presence of other sufu extracts (Table 1).

In contrast to mold-fermented sufu, the enzyme-ripened sufu examined in the present study is manufactured by soaking the non-fermented salted tofu cubes in a soybean-rice koji mash in a bottle. The koji mash is prepared by fermenting the mixture of soybean and rice with koji mold, *A. oryzae*. After ripening, the bottle containing sufu cubes and koji granules are distributed to market for sale. Therefore, the antiproliferative effect against cancer cells of the extract of koji granules was also examined in the present study. As shown in Table 2, the extract of the koji granules, regardless of ripening period, also exhibited an antiproliferative effect on Caco-2 and HT-29 cells. Similar to that observed with the sufu extract (Table 1), the antiproliferative effect of the koji extract generally increased as the dosage level and the ripening period increased. In the presence of 500 μg/mL of the 16-day koji granule extract, the Caco-2 and HT-29 cells showed the lowest viability of 78.66 and 75.61%, respectively (Table 2). This implied that ripening also enhanced the antiproliferative effect of the koji extract against the Caco-2 and HT-29 cells.

Soybeans were used as the raw material for the manufacture of tofu cubes, sufu and koji. The components of soybeans such as isoflavone, saponins, phytic acid, fiber and trypsin inhibitor have been shown to display anticancer activity[17-19]. Peptides derived from hydrolysates of soybean protein have also been reported to show cytotoxicities on tumor cell lines[20]. These compounds may all contribute to the antiproliferative activity of the extract of non-fermented tofu, sufu and koji granules on the Caco-2 and HT-29 cells as observed.

Previously, we noted that proteins were degraded into peptides and amino acids through the action of protease produced by *A. oryzae* during the ripening period. Sufu that has had an extended ripening period contained a higher content of amino nitrogen[7]. It is therefore reasonable to expect that these sufu and koji granules contain a larger amount of peptides, which possess antitumor cell activity, than the sufu or koji granules that had a shorter ripening period. Furthermore, other antitumor cell constituents might increase during the ripening period. For example, the content of bioactive isoflavones such as genestein and daizein increased in sufu as the ripening period extended[9]. Tofu before ripening contained 50.74 and 63.70 μg/ dried matter, of genistein and daidzein, respectively. These contents increased to 123.85 and 102.31 μg/ dried matter after 16 days of ripening. The increased content of these antitumor cell constituents may all result in the increased antitumor cell activity of sufu and koji granules with a longer ripening period (Tables 1 and 2). However, it cannot be ruled out that other antitumor bioactive compounds might be formed during the ripening period and thus contributed to the enhanced antitumor cell activity of the sufu and koji granules observed. This possibility merits further investigation.

II. *Effect of Sufu and Koji Granule Extracts on the 4-NQO-induced DNA Damage in Int-407 Cells*

4-NQO is a genotoxic agent that is capable of inducing DNA damage[21]. 4-NQO also possesses carcinogenic and mutagenic properties[22]. It is suggested that DNA damage induced by 4-NQO is an early event in carcinogenesis[23,24]. Irreversible topoisomerase I cleavage complex (Toplcc) induced recombination, and the covalent Toplcc produced by 4-NQO may play a role in 4-NQO carcinogenicity[25]. In the present study, the effects exerted by the sufu and koji granule extracts on the 4-NQO-induced damage in Int-407 cells were investigated using the single gel test or comet assay. With this technique, a small number of cells suspended in a thin agarose gel on a microslide are lysed, electrophoresed and stained with a fluorescent DNA-binding dye. Cells showed increased migration of chromosomal DNA from the nucleus toward the anode corresponded with increased DNA damage[26]. In the present study, comet score was obtained by summation of the number of cells in each class times the class number, yielding a rating between 0 (completely undamaged) and 200 (maximum damaged)[27]. As shown in Table 3, Int-407 in the absence of 4-NQO showed a score of 15.33 while a larger score of 166.33 was noted with cells treated with 4-NQO alone. This indicated that 4-NQO caused the DNA damage on intestine-407. Int-407 cells co-treated with 4-NQO and extract of the non-fermented tofu showed a score of 159.00, which is not significantly different ($p > 0.05$) from that of the cells treated with 4-NQO alone. On the other hand, a significantly lower score ($p < 0.05$) was noted with the cells treated simultaneously with 4-NQO and extract of sufu with a ripening period of 2h to 16 days when compared with that of control. Furthermore, the score of the 4-NQO-damaged cells was the least when co-treated with the extract of the

Table 2. Proliferation of colon cancer cells treated with methanol extracts of rice-soybean koji granule

Sample (μg/mL)	Proliferation (%)[a]					
	1	5	25	125	250	500
……. Caco-2 cell…….						
Control[b]	A100.00 ± 1.16a[c]	A100.00 ± 2.68a	A100.00 ± 0.54a	A100.00 ± 2.00a	A100.00 ± 2.48a	A100.00 ± 0.19a
koji granule	A97.63 ± 0.78a	A96.63 ± 2.47a	B95.81 ± 2.07a	B95.71 ± 1.93a	B95.46 ± 0.85a	B95.19 ± 2.56a
2 h-koji granule	C92.46 ± 2.44a	C91.62 ± 0.93ab	C89.96 ± 0.94abc	C89.15 ± 2.13bc	C89.07 ± 1.16bc	C87.83 ± 0.25c
8 day-koji granule	D87.20 ± 1.03a	D86.55 ± 0.53ab	D84.72 ± 2.21bc	D83.52 ± 0.53cd	D83.46 ± 0.36cd	D82.05 ± 0.87d
16 day-koji granule	E82.25 ± 0.24a	E81.97 ± 0.57a	E80.50 ± 0.04ab	E80.11 ± 2.01ab	E79.55 ± 0.67ab	E78.66 ± 2.64b
……HT-29 cell……						
Control	A100.00 ± 3.94a[c]	A100.00 ± 3.28a	A100.00 ± 3.19a	A100.00 ± 2.22a	A100.00 ± 3.82a	A100.00 ± 4.39a
koji granule	A98.37 ± 3.91a	A96.50 ± 1.74a	B93.44 ± 4.50ab	B93.22 ± 2.37ab	B91.02 ± 1.47b	B89.59 ± 0.21b
2 h-koji granule	BC91.62 ± 3.85a	B89.39 ± 2.93ab	C84.58 ± 3.20bc	CD83.19 ± 1.23c	C83.04 ± 3.30c	CD79.31 ± 2.32c
8 day-koji granule	CD87.68 ± 1.05a	CD85.84 ± 1.66ab	CD82.72 ± 1.20bc	D81.64 ± 3.44bc	CD81.39 ± 2.48bc	D77.83 ± 4.28c
16 day-koji granule	D84.38 ± 3.91a	D82.74 ± 1.36a	D80.49 ± 3.47ab	E78.77 ± 1.70b	D76.82 ± 2.14b	D75.61 ± 4.77b

[a]Proliferation was determined after 24 h of incubation and was expressed as percent of the control.
[b]Control is treated with PBS.
[c]Each value represents mean ± SD (n = 3). Means with different letters in the same row (a, b, c) and in the same column for the same cell line (A, B, C, D, E) were significantly different by Duncan's multiple range test ($p < 0.05$).

Table 3. Effect of methanol extracts of non-fermented tofu and sufu on the 4-NQO-induced genotoxicity in Intestine-407 cells

Sample[a]	Grade of damage in 50 cells					Comet score[b]
	0	1	2	3	4	500
Control[c]	35.67	13.33	1.00	0.00	0.00	E 15.33 ± 3.51[d]
4-NQO	0.00	3.00	3.33	18.00	25.67	A 166.33 ± 7.64
Non-fermented tofu extract +4-NQO	0.33	3.33	5.33	19.00	22.00	AB 159.00 ± 6.00
2 h-sufu extract +4-NQO	0.67	3.33	5.67	24.33	16.00	B 151.67 ± 4.04
8 day-sufu extract +4-NQO	3.00	6.33	14.67	18.00	8.00	C 121.67 ± 3.79
16 day-sufu extract +4-NQO	11.33	9.67	17.33	8.67	3.00	D 82.33 ± 2.89

[a]Sufu or non-fermented tofu extract and 4-NQO with a final concentration of 500 and 0.2 μg/mL, respectively, was examined.
[b]The total score for 50 comets were obtained by multiplying the number of cells in each class by the damage class, ranging from 0 to 200.
[c]Control is tested without 4-NQO and extracts.
[d]Each value represents mean ± SD (n = 3). Means with different letters in the same column (A, B, C, D, E) were significantly different by Duncan's multiple range test ($p < 0.05$).

Table 4. Effect of methanol extracts of rice-soybean koji granule on the 4-NQO-induced genotoxicity in Intestine-407 cells

Sample[a]	Grade of damage in 50 cells					Comet score[b]
	0	1	2	3	4	500
Control[c]	35.67	13.33	1.00	0.00	0.00	E 15.33 ± 3.51[d]
4-NQO	0.00	3.00	3.33	18.00	25.67	A 166.33 ± 7.64
koji granule +4-NQO	0.00	2.33	3.67	20.00	24.00	A 165.67 ± 3.06
2 h-koji granule +4-NQO	1.67	3.00	6.67	15.33	23.33	B 155.67 ± 5.03
8 day-koji granule +4-NQO	5.67	5.00	6.67	17.00	15.67	C 132.00 ± 6.56
16 day-koji granule +4-NQO	9.00	9.00	17.33	8.67	6.00	D 93.67 ± 2.52

[a] Rice-soybean koji granule and 4-NQO with a final concentration of 500 and 0.2 μg/mL, respectively, was examined.
[b]The total score for 50 comets were obtained by multiplying the number of cells in each class by the damage class, ranging from 0 to 200.
[c]Control is tested without 4-NQO and extracts.
[d]Each value represents mean ± SD (n = 3). Means with different letters in the same column (A, B, C, D, E) were significantly different by Duncan's multiple range test ($p < 0.05$).

16 day-sufu. This demonstrated that the anti-genotoxic effect exerted by sufu extract enhanced as the ripening period was extended with the 16 day-sufu extract exhibiting the highest anti-genotoxic activity.

Similar to that observed on sufu extract, the extracts of koji granules, collected at various sufu ripening periods, were also found to reduce the genotoxicity of 4-NQO on Int-407 cells (Table 4). A reduced score, ranging from 93.67 to 155.67, was noted with the 4-NQO-damaged cells co-treated with the ripened koji granule extract. Among the various koji granule extracts examined, the extract of koji granules collected after 16 days of ripening exhibited the highest anti-genotoxicity effect as the score of the 4-NQO-damaged cells was the lowest when co-treated with the 16 day-koji extract (93.67). Consistent with findings reported on Chungkook-jang, a traditional Korean fermented soybean product[28], this study clearly demonstrated that the sufu and the ripened koji granules have antigenotoxic activity.

Mutations occur when cells with damaged DNA divide[29]. The anti-genotoxigenic activity of the sufu extract, which reduced the level of damaged DNA observed (Tables 3 and 4), may account for the reduced mutagenicity of 4-NQO observed in the presence of the sufu extract as we reported previously[11]. It is likely that the reported anticancer constituents such as phytic acid, isoflavone, trypsin inhibitor[18-20] and the possible formation of other active compounds during the ripening period, may play an important role contributing to the antigenoxicity activity of the sufu and koji extracts observed. Furthermore, sufu that had an extended ripening period contained higher amount of genistein and daidzein[9], which have been shown to have greater antimutagenicity than their corresponding flavonoid glucosides[30]. These causes may thus contribute in part to the enhanced antigenotoxic activity of the ripened sufu observed in the present study. However, further investigation is required to characterize and elucidate the exact active constituents of sufu and koji granules that are responsible for reducing the genotoxicity of 4-NQO.

Data obtained from the present study showed that ripened sufu and the koji granules possess anticancer activity. In addition, they also exhibit a protective effect against the genotoxicity of 4-NQO on Int-47 cells. These effects were increased with the extension of the ripening period. Because of these properties, as well as sufu's increased aglycone content[9], ripened sufu and koji granules can be considered a functional seasoning that not only provide a characteristic pleasant flavor and aroma, but also serve to benefit human health.

ACKNOWLEDGMENTS

This research was financially supported by The National Science Council, ROC (Taiwan). (NSC 97-2313-B-002-015-MY3).

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