J. Korean Soc. Food Nutr.
21 (4), 454 ~ 459 (1992)

한국영양식량학회지

Chromosomal Aberrations in Chinese Hamster Ovary Cells Induced by Kojic Acid

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Abstract

Kojic acid, a fungal metabolite produced by some species of *Aspergillus* and *Penicillium*, was found to induce chromosomal aberrations in Chinese hamster ovary cells in the presence or absence of the rat liver homogenate (S9 mix). All categories of chromosomal aberrations increased with increased doses of kojic acid. Based on the this result, kojic acid was assumed to be a kind of mutagens. On the potential toxicity of this compound it becomes evident that kojic acid would not be used as a food additive at this time.

Key words : kojic acid, chromosomal aberration, mutagen, toxicity, food additive

INTRODUCTION

Kojic acid (5-hydroxymethyl-γ -pyrone), the antibacterial metabolite[1] produced by some species of *Aspergillus* and *Penicillium*[2] and many fungal strains isolated from the fermented Japanese food stuffs[3], was shown to inhibit mushroom tyrosinase[4] and the polyphenoloxidase (PPO) purified from potato, Florida spiny lobster, white shrimp, and grass prawn[5]. A Japanese product containing kojic acid, ascorbic acid, and citric acid is used as an inhibitor of tyrosinase in foods.

The use of kojic acid as a food additive to prevent enzymatic browning due to PPO should not ignore the safety of this compound. Only limited information is available related to kojic acid toxicity. The compound was reported to have a minimal lethal dose (MLD) of 30mg in 17g mice when given intraperitoneally[1]. The LD_{100} of the compound for 12-day old chick embryos in 12mg/egg[6]. Dogs receiving kojic acid intravenously at 150mg/kg body weight showed signs of intoxication, and died at 1g/kg[7]. Kojic acid was shown to cause DNA breaks and clastogenic effect in cultured rat liver cells[3,8], and to induce gene mutation in *Salmonella typhimurium*[9]. In addition, this compound was shown to induce a teratogenic effect in chick embryos[3]. However, kojic acid was shown not to induce "Rec effect" with *Bacillus subtilis*[10]. The compound also gave negative results in inducing dominant lethal effect in mice[9]. Auffray and Boutibonnes[11] showed that kojic acid was unable to induce SOS in *Escherichia coli*.

Due to these inconsistent and controversial results on kojic acid toxicity, this study was undertaken to assess the mutagenic and clastogenic activity of kojic acid in inducing sister chromatid exchange (SCE) and chromosomal aberrations (CAb) in Chinese hamster ovary (CHO) cells. SCE and CAb are used routinely to assess the potencies of chemical carcinogens/mutagens[12]. In addition, the Ames *Salmonella*/microsome assay was performed to confirm the mutagenic activity of kojic acid using both plate-incorporation and preincubation methods.

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MATERIALS AND METHODS

Cells

Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown as monolayers in medium 199 (M-199, Gibco Co., Grand Island, NY) supplemented with 10% fetal calf serum (FCS), and 1% antibiotic-antimycotic. The cultures were maintained at 37°C in a 5% CO_2, humidified atmosphere. Mycoplasma-free cultures at about 4×10^5 cells per T25-Cm^2 flask were maintained in 5ml medium for 24hr before addition of test compounds.

Chemicals

Kojic acid (Sigma, St. Louis, MO) was dissolved in distilled water or M-199. Cyclophosphamide (CP, Sigma) and triethylenemelamine (TEM, Polysciences Inc., Warrington, PA) were used as positive controls. The test compounds were freshly prepared each time.

Cytotoxicity test

Exponentially growing cells in 60×15mm culture dishes (Corning Laboratory, Houston, TX) were incubated for 24hr with different concentrations of kojic acid (1.5~12mg/ml) in M-199 in order to establish the 50% toxic concentration (TC_{50}) in this system. At the end of the exposure period, cultures were examined morphologically for cytopathic effects (CPE) using a phase contrast microscope (200 x magnification).

Following morphological examination and removal of the medium, the cells were washed twice with 0.1N NaOH. Aliquots were removed for protein determination using the Lowry assay method[13]. After the percentage decrease of total cell protein at each test concentration was determined, the TC_{50} for kojic acid in CHO cells was calculated.

Determination of chromosomal aberrations (CAb)

Exponentially growing cells in T25-Cm^2 flasks were incubated for 2 hr with kojic acid at 3, 4, 5 and 6mg/ml in M-199 and controls with or without the S9 mix. The rat hepatic S9 mix was prepared following the methods of Ames *et al.*[14] After incubation, cells were washed with PBS and incubated for another 24hr in fresh medium. Colchicine (0.1 μg/ml) was added for the last 3 hr of culture. The positive controls include CP in the presence of the S9 mix, and TEM in the absence of the S9 mix.

Chromosome preparations

Three hours after the addition of colchicine, cells were harvested by mitotic shake-off[15], then centrifuged and treated for 20 min with a hypotonic solution (75mM KCl). Cells were then fixed twice for 20 min each with a methanol-glacial acetic acid (3 : 1, v/v) fixative, dropped onto clean cold slides and air dried. A modified fluorescence plus Giemsa technique[16] was used for differential staining of sister chromatids.

For scoring CAb, slides were stained in 4% Giemsa in Gurr buffer and permanently mounted. At least one hundred metaphases per flask were scored for each dose; individual types of aberrations including gabs, breaks, deletions, exchanges, rings and dicentrics were scored.

RESULTS AND DISCUSSION

Kojic acid at high doses (9mg/ml and above) was toxic to CHO cells; morphological cytopathic effects observed included rounding of cells and loss of nucleolar definition. From the loss of cellular proteins, the TC_{50} level of kojic acid in CHO cell system was determined to be 10.86 ± 3.86 mg/ml culture medium.

Kojic acid also caused a dose-related increase in chromosomal aberrations (CAb) in CHO cells in the presence or absence of the liver homogenate (S9 mix) that is a hepatic activator. Except for rings, all categories of chromosomal aberrations increased with increased doses of kojic acid in the absence of the liver homogenate (Table 1). Kinosita *et al.*[3]

Table 1. Chromosomal aberrations induced by kojic acid in Chinese hamster ovary cells in the absence of the hepatic activation system[1]

Treatment	Dose (μg/ml)	Aberrant cells (%)	Number of aberrations per 100 metaphases						
			Gabs	Breaks	Deletions	Exchanges	Rings	Dicentrics	Total aberrations
Control	0	20[d][3]	11[a]	0[c]	5[c]	5[a]	1[a]	3[c]	25[d]
TEM[2]	0.25	42[a]	21[a]	4[ab]	12[a]	9[ab]	3[a]	8[b]	57[a]
Kojic acid	3,000	29[c]	16[a]	3[b]	5[bc]	7[b]	2[a]	6[bc]	39[c]
	4,500	35[b]	18[a]	4[ab]	8[b]	12[a]	2[a]	7[b]	51[b]
	6,000	46[a]	23[a]	5[a]	11[a]	12[a]	1[a]	12[a]	64[a]

[1] Media were not included rat liver homogenate, S9 mix. Aberration investigation was based on Evans's description[18]
[2] Triethylenemelamine
[3] Numbers within the same columns followed by different letters are significantly different according to Duncan's multiple range test at $p<0.05$

Table 2. Chromosomal aberrations induced by kojic acid in Chinese hamster ovary cells in the presence of the hepatic activation system[1]

Treatment	Dose (μg/ml)	Aberrant cells (%)	Number of aberrations per 100 metaphases						
			Gabs	Breaks	Deletions	Exchanges	Rings	Dicentrics	Total aberrations
Control	0	19[c][3]	5[c]	4[b]	3[c]	4[b]	3[a]	11[a]	30[e]
CP[2]	5	43[b]	26[a]	6[ab]	10[a]	7[b]	6[bc]	7[ab]	62[b]
Kojic acid	3,000	38[b]	19[b]	8[a]	5[b]	5[b]	8[ab]	4[b]	49[d]
	4,500	42[b]	25[a]	4[b]	3[c]	7[b]	9[ab]	9[a]	57[c]
	6,000	49[a]	28[a]	7[a]	4[bc]	12[a]	11[a]	8[ab]	70[a]

[1] Media were included rat liver homogenate, S9 mix. Aberration investigation was based on Evans's description[18]
[2] Cyclophosphamide
[3] Numbers within the same columns followed by different letters are significantly different according to Duncan's multiple range test at $p<0.05$

found both asymmetric mitosis and chromosome stickiness and breaks in cultured rat liver cells after treatment with kojic acid. Wei *et al.*[17] reported that kojic acid solution at 80μg/ml was shown to inhibit approximately 85% of the mushroom tyrosinase activity.

In the presence of the liver homogenate, kojic acid also produced all categories of CAb (Table 2). There were more gabs, exchanges and rings than the other CAb types. However general trend of mutation in this case was similar to one of mutation in the absence of the liver homogenate, except ring type.

The types of chromosome damage which can be cytologically distinguished at metaphase can be divided into two main groups: chromosome type and chromatid type. The circulating lymphocyte is in the G_0 or G_1 phase of mitosis and exposure to ionising radiations and certain other mutagenic agents during this stage produces chromosome type damage where the unit of breakage and reunion is the whole chromosome. However, cells exposed to these agents while in the S or G_2 stages of the cell cycle, after the chromosome has divided into two sister chromatids, yield chromatid type aberrations and only the single chromatid is involved in breakage or exchange[18]. In this study we could demonstrate that kojic acid makes all damage types of chromosome

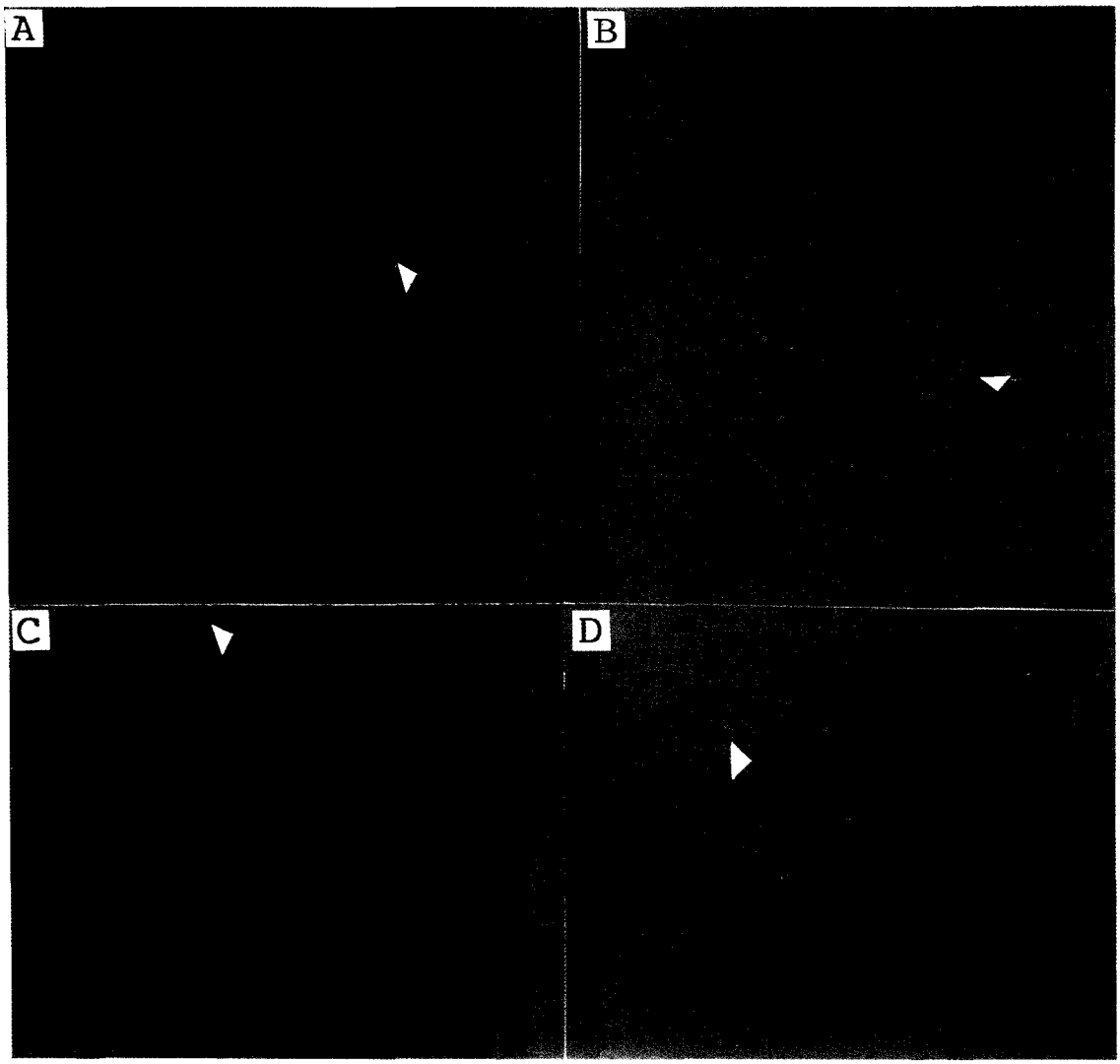

Fig. 1. Some typical types of chromosomal aberrations in Chinese hamster ovary cells induced by kojic acid.
A : Break, B : gab, C : terminal deletion, D : dicentric

and chromatid such as dicentic, terminal deletion, exchange, ring, gap and break.

Human intoxication from consumption of oriental fermented foods containing kojic acid has not been reported. However, the results of this study demonstrate that kojic acid is capable of inducing CAb in cultured CHO cells (Fig. 1). It is assumed that this compound can be functioned as a mutagen in cultured Chinese hamster cells. Furthermore, this compound was demonstrated to induce mutations in *Salmonella typhimurium* strains TA98 and TA 100 using both plate-incorporation and preincubation methods[19]. In addition, kojic acid caused dose-related and significant increases of sister chromatid exchange (SCE) frequency in CHO cells with or without the liver homogenate (S9 mix). The binding of kojic acid to constituents of the S9 mix to decrease the availability of the compound for reaction with the genetic makeup of the cells may have resulted in reduction of SCE frequency in the group

with the addition of the S9 mix. The decrease of SCE frequency in the group with kojic acid at 6mg/ml could be due to the toxicity of the compound[19].

Based on this result and the literature reports on the potential toxicity of this compound, it becomes evident that kojic acid would not be approved for use as a food additive at this time. Further studies should be conducted to elucidate the mutagenic and clastogenic activity and the potential carcinogenic of this compound in order to verify its safe use as a food additive in preventing enzymatic browning in agricultural products.

ACKNOWLEDGEMENT

This research was carried out during the oversea research supported by the Ministry of Education of the Korean Government in 1990.

REFERENCES

1. Morton, H. E., Kocholaty, W., Junowicz-kocholaty, R. and Kelner, A. : Toxicity and antibiotic activity of kojic acid produced by *Aspergillus luteovirescens*. *J. Bacteriol.*, **50**, 579 (1945)
2. Parrish, F. W., Wiley, B. J., Simmons, E. G. and Long, L. Jr. : Production of aflatoxins and kojic acid by species of *Aspergillus* and *Penicillium*. *Appl. Microbiol.*, **14**, 139 (1966)
3. Kinosita, R., Ishiko, T., Sugiyama, S., Seto, T., Igarasi, S. and Goetz, I. E. : Mycotoxins in fermented food. *Cancer Res.*, **28**, 2296 (1968)
4. Saruno, R., Kato, F. and Ikeno, T. : Kojic acid, a tyrosinase inhibitor from *Aspergillus albus*. *Agric. Biol. Chem.*, **43**, 1337 (1979)
5. Chen, J. S., Wei, C. I., Rolle, R. S., Otwell, W. S., Balaban, M. O. and Marshall, M. R. : Inhibitory effect of kojic acid on some plant and crustacean polyphenol oxidases. *J. Agric. Fd. Chem.*, **39**, 1396 (1991)
6. Lee, H. F., Boltjes, B. and Eisenman, W. : Kojic acid as an inhibitor of tubercle bacilli. *An. Rev. Tuberc.*, **61**, 738 (1950)
7. Friedemann, T. E. : Chemical and physiological properties of kojic acid. *Science*, **80**, 34 (1934)
8. Stark, A. : Mutagenicity and carcinogenicity of mycotoxins: DNA binding as a possible mode of action. *Ann. Rev. Microbiol.*, **34**, 235 (1980)
9. Shibuya, T., Murota, T., Sakamoto, K., Iwahara, S. and Ikeno, M. : Mutagenicity and dominant lethal test of kojic acid. *J. Toxicol. Sci.*, **7**, 255 (1982)
10. Boutibonnes, P., Auffray, Y., Malherbe, C., Kogbo, W. and Marias, C. : Proprietes antibacteriennes et genotoxiques de 33 mycotoxines. *Mycopathologia*, **87**, 43 (1984)
11. Auffray, Y. and Boutibonnes, P. : Evaluation of the genotoxic activity of some mycotoxins using *Escherichia coli* in the SOS spot test. *Mutat. Res.*, **171**, 79 (1986)
12. Abe, S. and Sasaki, M. : SCE as an index of mutagenesis and/or carcinogenesis. In *"Sister chromatid exchange"*, Sandberg, A. A. (ed.), Alan R. Liss Company, New York, p.461 (1982)
13. Lowry, O. H., Rosebrough, W. J., Farr, A. L. and Randall, R. J. : Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265 (1951)
14. Ames, B. N., McCann, J. and Yamasaki, E. : Methods for detecting carcinogens and mutagens with the *Salmonella*-microsome mutagenicity test. *Mutat. Res.*, **31**, 347 (1975)
15. Terasima, T. and Tolmach, L. J. : Change in X-ray sensitivity of HeLa cells during the division cycle. *Nature*, **190**, 1210 (1961)
16. Goto, K., Maeda, S., Kano, Y. and Sugiyama, T. : Factors involved in differential staining of sister chromatids. *Chromosoma*, **66**, 351 (1978)
17. Wei, C. I., Huang, T. S., Chen, J. S., Marshall, M. R. and Chung, K. T. : Production of kojic acid by *Aspergillus candidus* in three culture media. *J. Food Prot.* (in press, 1992)
18. Evans, H. J. : Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. In *"Handbook of mutagenicity test procedures"*, Kilbey B. J., Legator M., Nichols W. and Ramel C. (eds.), Elsevier Science Publishers, BV, p. 405 (1984)
19. Wei, C. I., Huang, T. S., Fernando, S. Y. and Chung, K. T. : Mutagenicity studies of kojic acid. *J. Food Prot.*, (in press, 1992)

(1992년 6월 20일 접수)

Kojic Acid에 의해 유기된 Chinese Hamster 난소세포의 염색체 변이

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요 약

수종의 *Aspergillus*속 및 *Penicillium*속 균이 생산하는 진균대사 산물인 kojic acid가 독성분해 활성제의 첨가유무에 관계없이 Chinese hamster 난소세포에 염색체 변이를 일으키는 것이 확인되었다. Kojic acid 처리량의 증가에 따라 염색체 변이 또한 증가되었다. Kojic acid의 잠재독성에 관한 본 실험결과에 기초해 이는 일종의 변이 유기체로 추정되며 현재로는 식품첨가제로서의 사용에 문제가 있는 것으로 사료된다.