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In vivo Genotoxic Potential of Kojic Acid in Rodent Multiple Organs Detected by the Comet Assay

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Kojic acid has been used for skin whitening as a cosmetic agent. Kojic acid is believed to be hepatocarcinogenic in mice. We conducted the comet assay in mouse and rat multiple organs to evaluate its *in vivo* genotoxic potential. Kojic acid induced dose-dependent DNA damage in ddY mouse stomach and liver and in Wistar rat stomach, liver, lung, and bone marrow after a single gavage administration at ≦1000 mg/kg. Its hepatic genotoxicity detected by the comet assay seems to contradict to absence of its hepatic tumor initiating activity revealed by the two-step carcinogenesis studies with mice and rats. However, considering the possibility that the sensitivity of the two-step carcinogenesis studies performed are not high enough to detect weak initiating activity of a chemical, it would be premature to conclude that its hepatic genotoxicity contradicts to the absence of initiating activity. In mice fed a diet containing 3% kojic acid for up to 10 days, DNA migration increased in the stomach after feeding for 2 days but it did not increased after feeding for 1 day and ≧4 days. In the colon, DNA migration after feeding of 3% kojic acid for 2 days was higher than the control values, but this increase was not statistically significant. In the stomach and colon, any statistically significant increases in DNA migration were not observed after feeding of 1.5% kojic acid. In the liver, DNA migration increased with feeding period and the increases after feeding of 3% and 1.5% kojic acid for ≧4 days and 6 days, respectively, were statistically significant. Our present results suggested good correlation between hepatocarcinogenicity of kojic acid and increasing tendency of its hepatic genotoxicity with dosing period when it is given to mice continuously in the diet.

Key words: kojic acid, hapatocarcinogenicity, DNA damage, comet assay, gavage, feeding

Introduction

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone; CAS No. 501-30-4) is a secondary metabolic product from various species of *Aspergillus* and *Penicillium* (1,2). It inhibits polyphenoloxidase (tyrosinase), which catalyzes the conversion of tyrosine to melanin *via* 3,4-dihydroxyphenylalanine and dopaquinone (3,4) in mushrooms (3), potatoes, apples and crustaceans (5). Kojic acid was used as an inhibitor of polyphenoloxidase in foods to prevent enzymatic browning of raw crabs and shrimps. Although kojic acid is not used as a food additive now, it is mainly used as a cosmetic agent for skin whitening properties (6,7) because of its inhibitory actions on human melanocyte tyrosinase (8). Kojic acid which is known to be genotoxic without microsomal activation, inducing mutation in bacteria and mammalian cells, chromosome aberrations in mammalian cells, and DNA damages detected by comet assay in mammalian cells (9,10) has been reported to induce hepatic tumors in mice (11), although no long-term carcinogenesis studies in rats have been reported. The initiation activity in the liver has been investigated in two step carcinogenesis studies of rats and mice. In this study, 2% kojic acid orally given to F344/DuCrj rats for 4 weeks followed by partial hepatectomy during administration of a tumor promoter, phenobarbital, was considered not to possess initiation activity of hepatocarcinogenesis (12). Similarly, no liver tumor initiating activity of kojic acid was found in mice, with feeding a diet containing 3% kojic acid at initiation step and phenobarbital at promotion step with partial

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hepatectomy (13). In these studies, partial hepatectomy was performed two weeks after the cessation of tumor initiator administration. It is considered this protocol might be unsuitable to detect weak initiating activity of a chemical (personal communication by Dr. Kunitoshi Mitsumori, Tokyo University of Agriculture and Technology). Indeed, the incidences of hepatocellular adenomas increased in the 1.5% and 3% groups [7/10 and 5/10 in *p53*(+/−) mice and 2/12 and 5/12 in *p53*(+/+) mice, respectively], in comparison with findings in the control group [0/7 in *p53*(+/−) mice and 0/10 in *p53*(+/+) mice] (14). These facts suggest the possibility that kojic acid acts on hepatocytes as an initiator in mice and leads to the induction of hepatocelluar tumors. Although kojic acid induced micronuclei in the liver of regenerating mouse liver (9), no micronuclei were observed in regenerating rat liver and infant rat liver without partial hepatectomy (15).

The main purpose of this study is to evaluate the *in vivo* genotoxicity in multiple organs of mouse and rat using the comet assay.

Materials and Methods

Animals and chemicals: Male ddY mice and male Wistar rats were obtained from SLC Japan Co. (Shizuoka, Japan) at 7 and 5 weeks of age, respectively, and they were used after 1 week of acclimatization. They were fed powdered basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum throughout the acclimatization and experiment periods. Four animals per cage were housed in an air-conditioned animal room. The animal room was at 20–24°C and 50–70% humidity with a 12-h light-dark cycle.

Kojic acid kindly provided by Alps Pharmachemical Industry Co., Ltd. (Gifu, Japan) was suspended in 0.5 % sodium carboxymethylcellulose (CMC-Na) aqueous solution at 50 and 100 mg/mL or admixed into a powdered CRF-1basal diet at 1.5 or 3%. The diet was prepared just before feeding. To set the appropriate dose of Kojic acid for each study by single gavage administration, we determined the approximate maximum tolerated dose (MTD) for each species by simple acute toxicity experiments.

Comet assay in mice and rats: Four 8-week-old mice per group or four 7-week-old rats per group were treated with kojic acid by giving a single gavage administration at up to 1000 mg/kg (MTD for both species). This experiment followed the design of our previous comet assay studies on multiple mouse organs (16–18). From shortly after they were treated until just before they were killed, the animals were carefully observed for pharmacotoxic signs and they were killed by bleeding under anesthesia 3 or 24 h after the treatment, and then 8 organs-glandular stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow-were removed.

In another experiment, four 8-week-old mice per group were fed a diet containing 0, 1.5 and 3% kojic acid for up to 10 days (mean body weight at starting was 37.8 *g*). This is based on the previous study which demonstrated that 1.5 and 3% kojic acid in the diet increased the incidences of hepatocellular adenomas and no visible alteration in the health of the mice during the exposure to kojic acid (14). Slides for comet observation were prepared after the feeding for 1, 2, 4, 6, and 10 days as described below. In the feeding study with mice, feeding started at 9:00am, mice were sacrificed by bleeding under anesthesia at 9:00am, and daily diet intake and body weight were measured at every 9:00am.

The liver, kidney, lung, and brain were minced, suspended in 4 mL chilled homogenizing solution (pH 7.5) containing 0.075 M NaCl and 0.024 M Na_2EDTA, and then homogenized gently using a Potter-Elvehjem type homogenizer at 500–800 rpm, in ice (16). The glandular stomach, colon, and urinary bladder were opened and rinsed with physiological saline; then the mucosa was scraped into 4 mL chilled homogenizing buffer and homogenized gently using a Potter-Elvehjem type homogenizer at 500–800 rpm, in ice. To obtain nuclei, the homogenate was centrifuged at 700 *g* for 10 min at 0°C, and the precipitate was re-suspended in chilled homogenizing buffer at 1 *g* organ weight per milliliter (16). Slides prepared from nuclei isolated by homogenization were placed in a chilled lysing solution as described above, and then in chilled alkaline solution (300 mM NaOH and 1 mM Na2EDTA, pH 13) for 10 min in the dark at 0°C. Electrophoresis was conducted at 0°C in the dark for 15 min at 25 V (0.96 V/cm) and approximately 250 mA. The slides were neutralized and thereafter stained with 50 μL of 20 μg/mL ethidium bromide. Photographs of comet images were taken using Fuji Neopan Presto 400 Black & White film at 200× magnification with the aid of a fluorescence microscopy and the length of the whole comet (''length'') and the diameter of the head (''diameter'') were measured manually using a scale for 50 nuclei per organ per animal. We calculated migration as the difference between length and diameter. Mean migration of 50 nuclei from each organ was calculated for each individual animal. The differences between the averages of four treated animals and the untreated control animals were compared using Dunnett test after one-way ANOVA. A *p*-value less than 0.05 was considered statistically significant.

Neutral diffusion assay: To evaluate the extent of cytotoxicity associated with the treatment, neutral diffusion assay was conducted. Incidence of nuclei with low molecular weight DNA was used as indicative of cell death. After incubation in the lysis solution for 1 h, one comet slides per sample were placed in chilled alkaline

solution (300 mM NaOH and 1 mM Na_2EDTA, pH 13) for 10 min in the dark at 0°C, and then neutralized and thereafter stained with 50 μL of 20 μg/mL ethidium bromide. The frequency of nucleus with diffuse DNA among 1000 nuclei was scored using a fluorescence microscopy at 200× magnification. The number of diffused nuclei was statistically analyzed using the chi-square test.

Results

Although oral gavage with kojic acid at 2000 mg/kg led to all mice and rats death within 3 h, any death and morbid sign were not observed at 1000 mg/kg by simple acute toxicity experiment. Therefore, the studies by a single gavage were carried out at ≦1000 mg/kg. The damage of DNA was indicated by DNA migration (Fig. 1, 2, and 3). In a single gavage treatment with kojic acid, dose-related DNA damage in mouse stomach and liver was observed at 3 or 24 h (Fig. 1). Kojic acid treatment increased DNA migration in rat stomach, liver, lung, and bone marrow, but the induction of DNA migration in the liver was less in rats than in mice (Fig. 2). No increase in the frequency of nuclei with diffused DNA was detected in the neutral diffusion assay (data not shown). Thus, DNA migration observed was not likely to be due to cell death.

In another experiment where mice were given kojic acid by feeding for up to 10 days, significantly increased DNA migration was observed in the stomach and liver (Fig. 3). In the stomach, DNA migration increased after feeding of 3% kojic acid for 2 days but it did not increase after feeding of 3% kojic acid for 1 day and ≧4 days. In the colon, DNA migration after feeding of 3% kojic acid for 2 days was higher than the control values, but this increase was not statistically significant. In the stomach and colon, any increases in DNA migration were not observed after feeding of 1.5% kojic acid. In the liver, on the other hand, DNA migration increased with feeding period and the increases after feeding of 3 % and 1.5% kojic acid for ≧4 days and 6 days, respectively, were statistically significant. No increase in the frequency of nuclei with diffused DNA was detected in the neutral diffusion assay (Fig. 4). Thus, DNA migration observed was not likely to be due to cell death.

As shown in Fig. 5, food intake and body weight gain in mice of 1.5% and 3% kojic acid groups were lower than those in control mice. Daily intake of kojic acid in mice of the 3% kojic acid group was >2-fold higher

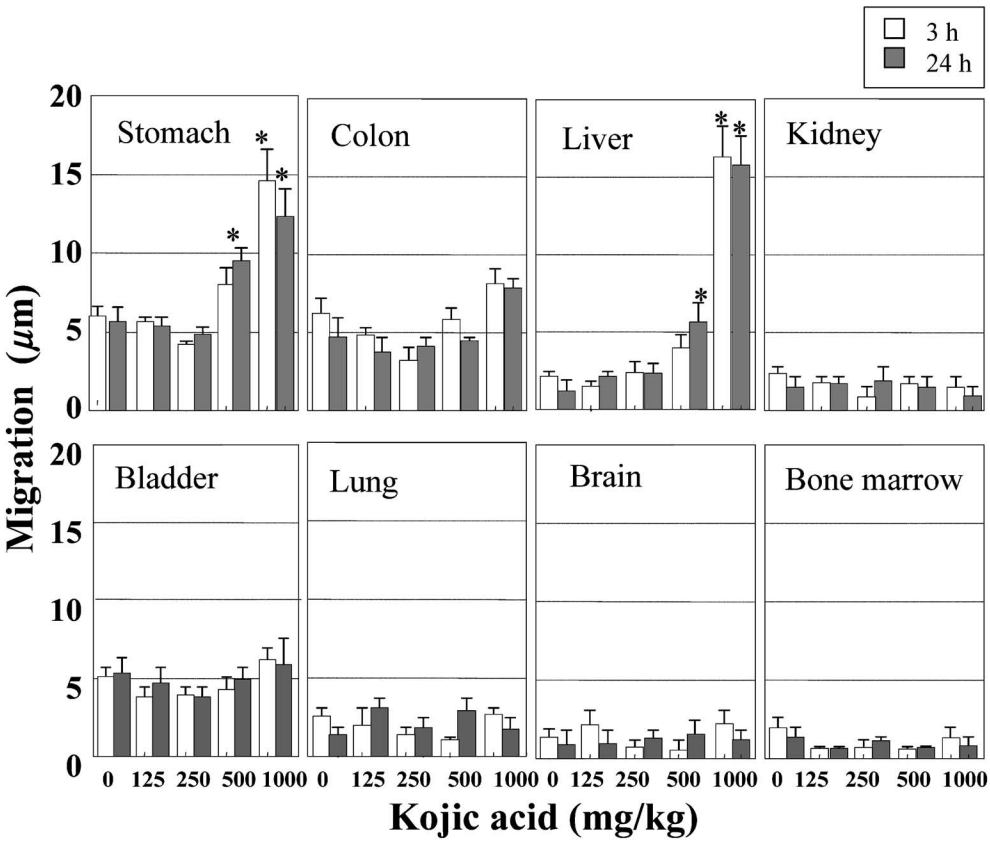

Fig. 1. DNA damage measured with the comet assay in organs from mouse treated by a single gavage with kojic acid. Data represent mean ± SEM of 4 animals. Statistical significant difference from untreated control: $^*p<0.05$.

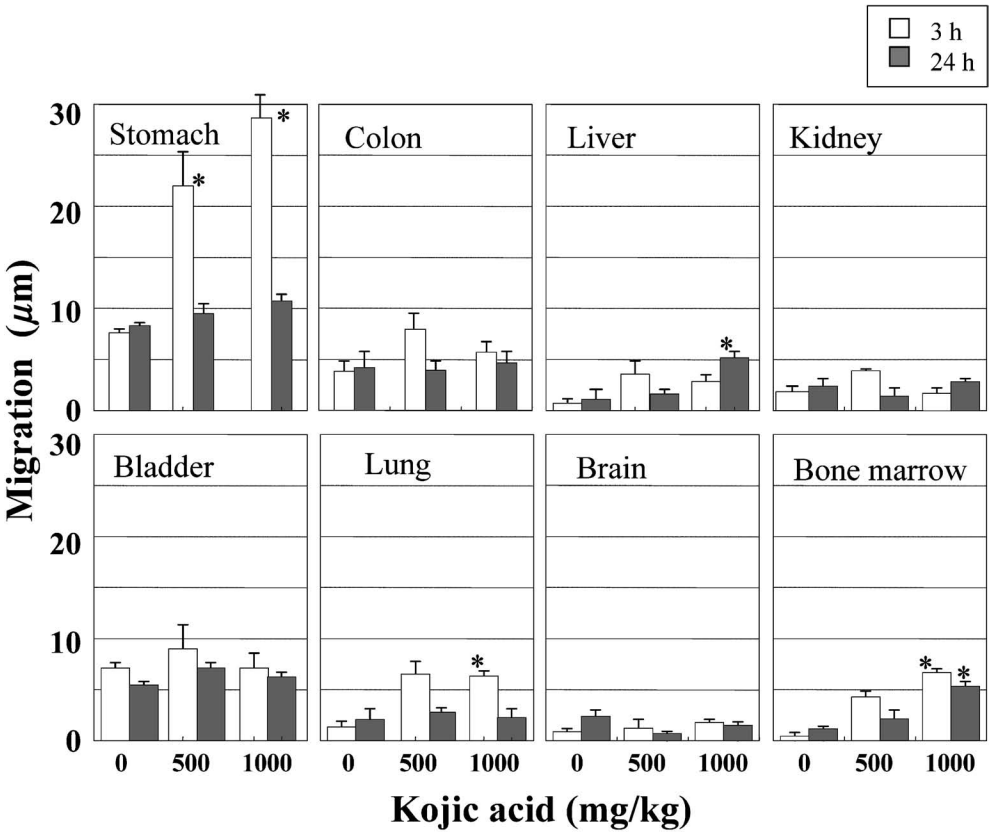

Fig. 2. DNA damage measured with the comet assay in organs from rat treated by a single gavage with kojic acid. Data represent mean ± SEM of 4 animals. Statistical Significant difference from untreated control: $^{*}p < 0.05$.

than the highest dose used for its gavage-administration (1000 mg/kg).

Discussion

Thyroid follicular cell adenomas were found to develop in male B6C3F$_1$ mice fed diet containing 1.5 or 3 % kojic acid for 20 months,although female B6C3F$_1$ mice receiving 3% kojic acid in the diet were also found to develop hepatocellular tumors in the first carcinogenesis experiment conducted by Fujimoto *et al.* (11). However, no initiating activity was detected in the two-step carcinogenesis studies in mice and also in rats received (12,13). Therefore, consistent interpretation or underlying mechanisms of liver tumorigenicity remain uncertain. In the present study, we demonstrated that kojic acid induced DNA damage in the liver of mouse and rat. This observation seems to contradict to the negative responses in the two-step carcinogenicity studies with both species. Considering that the modified two-step carcinogenesis study where animals receive a chemical after partial hepatectomy was recently conducted to detect more appropriately weak initiating activity of a chemical (20), no initiating activity of kojic acid in mice and rats previously reported (12,13) might not be evaluated appropriately. Therefore, it would be premature to conclude that its hepatic genotoxicity detected by the comet assay in both species contradicts to negative responses in the two-step carcinogenicity studies with both species. In our previous study, kojic acid induced micronuclei in regenerating liver of partially hepatectomized mice (9). Considering our previous observation that the comet assay-detectable DNA lesions can form chromosome aberrations in human lymphoblastoid WTK1 cells (10), it is interesting that both comet assay-detectable DNA damage and micronuclei were induced in mouse liver, which is a possible target organ of kojic acid carcinogenicity. On the other hand, DNA damages detectable by comet assay but not by micronuclei assay were induced in rat liver. This discrepancy might be explained by followings: the comet assay-detectable DNA lesions in the cells in proliferating phase might be able to form chromosome aberrations (micronuclei) as observed in lymphoblastoid WTK1 cells. The induction of the comet assay-detectable DNA lesions in mouse liver is high enough to form chromosome aberrations (micronuclei) under regenerating conditions but it is not so high in rat liver. This is supported by the present observation that the induction of DNA migration in the liver was less in rats than in mice (Figs. 1 and 2).

Although the intake of kojic acid for first 1 day of

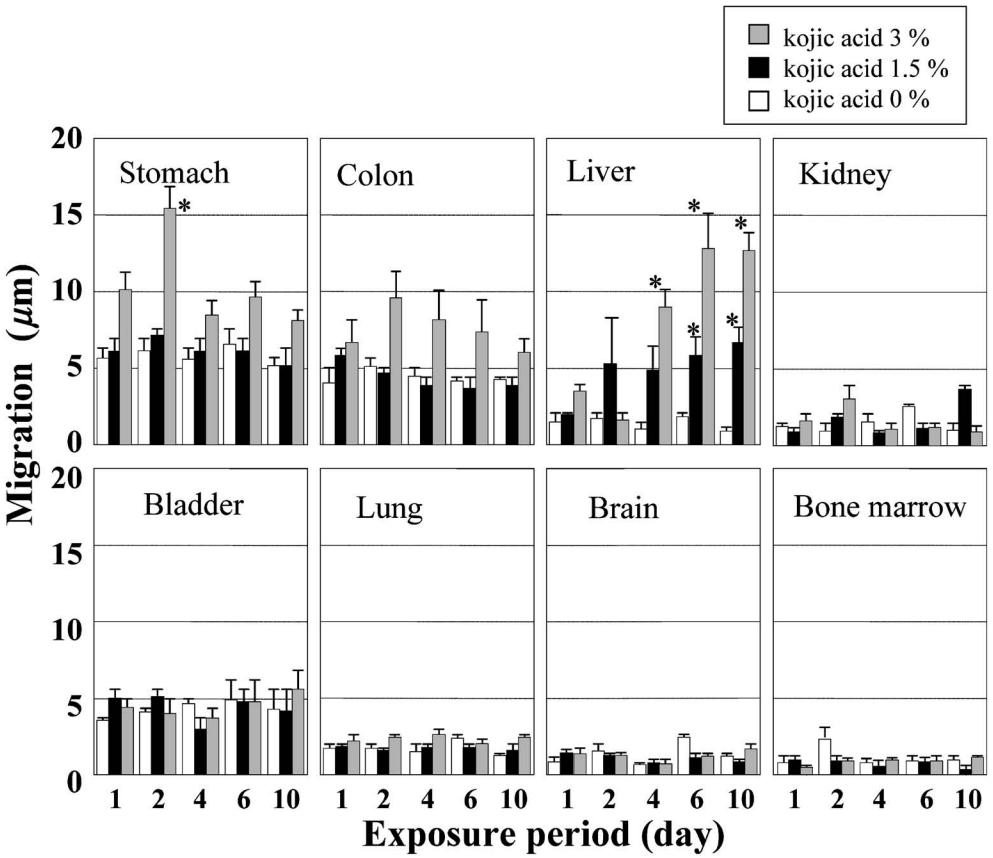

Fig. 3. DNA damage measured with the comet assay in organs from mice fed kojic acid for up to 10 days. Data represent mean ± SEM of 4 animals. Statistical significant difference from untreated control: $^{*}p < 0.05$.

feeding was higher than the highest dose (1000 mg/kg) used in the gavage study, the induction of DNA migration tended to be lower in mice received 1-day feeding of kojic acid than those received a single gavage of kojic acid. In the pharmacokinetics study with rats received a single gavage of kojic acid, it was shown that kojic acid was rapidly absorbed and metabolized, its plasma level peaked at about 2 h after its administration, and it returned rapidly to low levels at 6 h (21). Although species difference in pharmacokinetics of orally dosed kojic acid cannot be ruled out, it can be considered that orally given kojic acid is rapidly absorbed and metabolized in mice, like as in rats. Since rodents eat food well in night to early morning, the results in the feeding study would reflect mainly the effect of kojic acid fed at 0–6 h prior to sacrifice at 9:00 am (mid-night to morning) but not at 6–24 h prior to sacrifice at 9:00 (afternoon to mid-night). Although daily intake of kojic acid in mice of the 3% group was >2-fold higher than the highest dose used for its gavage-administration (1000 mg/kg), its intake in mid-night to early morning might not be higher than the dose used in the gavage study. In general, furthermore, gavage administration increases plasma levels of chemicals higher and transiently, and feeding administration increases the levels relatively low and continuously. Therefore, plasma levels of kojic acid in the feeding study might increase the levels of kojic acid up to lower level than that in the gavage study. Other possible explanation for the difference in genotoxicity between by feeding administation and gavage administration might be possible. Although it is remained for further studies whether gavage-administered kojic acid saturate metabolic/detoxification pathway of kojic acid, gavage-administered kojic acid may tend to saturate metabolic/detoxification pathway of kojic acid, like as Yuan *et al.* (22) showed in their study with benzyl acetate, which might result in gavage-administered kojic acid tended to be more genotoxic.

When kojic acid was given to mice in the diet, its genotoxicity tended to decrease as an administration period became long with the exception for its genotoxicity in the liver. In some cases, chemicals added to the food and/or water may cause it to become unpalatable to the animals, especially at high doses (23,24). Animals dosed in this manner may consume less food than they would if the chemicals were absent from the food and thus take in a smaller amount of chemical than planned (23,24). Decreases in food (caloric) intake that are often

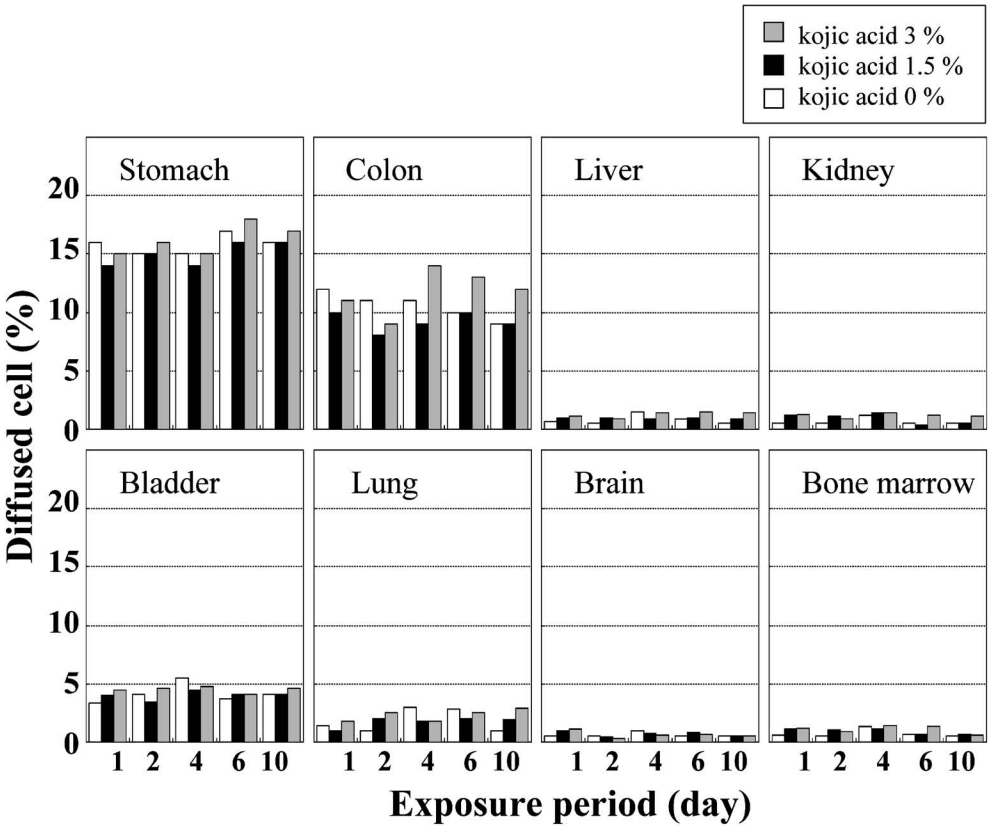

Fig. 4. Diffused cell frequency in organs from mice fed kojic acid for up to 10 days. Data represent mean of 4 animals.

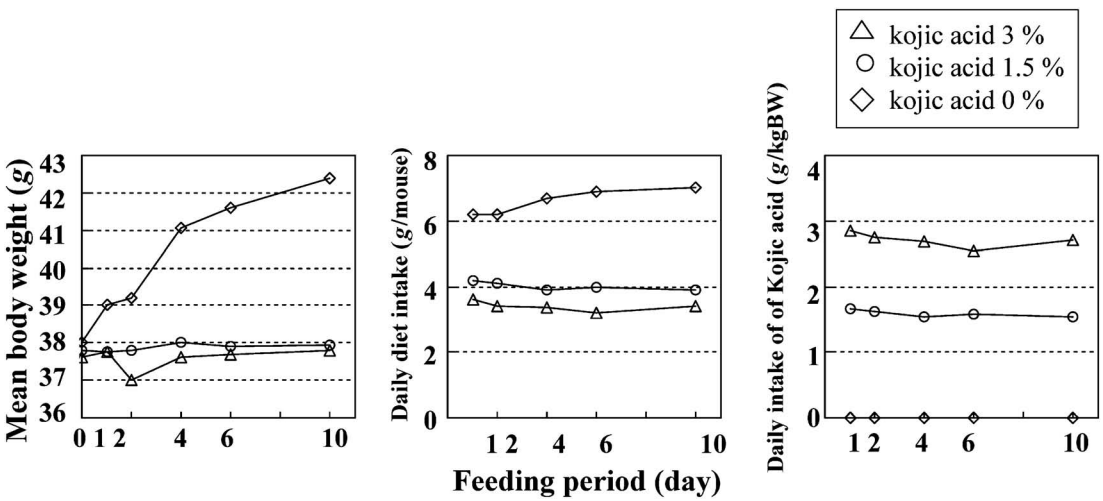

Fig. 5. Body weight change (left), daily intake of diet (center), and daily intake of kojic acid (right) in the feeding study with mice. Data represent mean of 4 animals.

associated with reductions in tumor burden and improvements in survival (23,24) might affect genotoxicity. In this study, as shown in Fig. 5, food intake and body weight gain in mice of the 1.5% and 3% groups were lower than those in control mice. Therefore, kojic acid may have some adverse effects on food digestion in mice. Our present observation that the genotoxicity of kojic acid decreased with dosing period in the stomach might be possibly explained by decreases in food (caloric) intake.

Since kojic acid is rapidly absorbed and metabolized, the increase in hepatic genotoxicity of feeding ad-

ministered kojic acid might be explained by the accumulation of damaged cells in this organ but not by accumulation of kojic acid in this organ. Damaged cells could not accumulate in the stomach mucosa that is a proliferating organ with turning-over of mucosa cells, which might lead to the decrease in gastric genotoxicity with feeding period. Our present result suggested good correlation between its hepatocarcinogenicity and increasing tendency of its hepatic genotoxicity with dosing period when it is given to mice continuously in the diet. In a consensus of minimal standards for obtaining reproducible and reliable comet data at the International Workshop on Genotoxicity Test Procedures (IWGTP) in 1999 (18), it is recommended that test substances are generally administered as a single treatment at high dose level which should be sufficient high to elicit signs of toxicity. The gavage route may bear little, if any, resemble to human exposures to chemicals used as food additive and/or cosmetics such as kojic acid (25). In the U.S.A., more than 3000 total substances together comprise an inventory often referred to as ''Everything'' Added to Food in the United States (EAFUS) (26). The distribution of EAFUS rodent carcinogenesis and noncarcinogenesis revealed 17 noncarcinogens and 1 carcinogen among chemicals administered in the feed, compared with 7 noncarcinogens and 16 carcinogens among gavage-administered chemicals (23). Thus, the distribution clearly shows that noncarcinogens are significantly more prevalent in feed studies compared with gavage studies and that the use of non-feed routes may tend to increase the likelihood of a positive rodent cancer-test response (23). Saturation of metabolic/detoxification pathway may explain why gavage-administered chemicals generally tended to be more carcinogenic (23). However, for real-life dose levels in humans, saturation may seldom occur (23). Like as in rodent cancer-test response, present results showed that gavage-kojic acid tended to be more genotoxic. If higher response of its genotoxicity in gavage studies could be explained by saturation of metabolic/detoxification pathway, genotoxic response by continuous dosing might be more significant toxicologically. The comet assay by continuous dosing of kojic acid in the diet would give useful information to predict its hepatocarcinogenicity.

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