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Induction of Thyroid Tumours in (C57BL/6N × C3H/N)F_1 Mice by Oral Administration of Kojic Acid

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Abstract—The tumorigenicity of kojic acid (KA), which is widely used for food and cosmetics in Japan, was examined in B6C3F_1 mice. Female and male animals were divided into three groups and given 0, 1.5 and 3.0% KA containing food from the age of 6 weeks. At sacrifice after 20 months, thyroid weights were significantly increased in both sexes of mice receiving KA, especially in the male groups. The enlarged thyroid glands histologically featured diffuse hyperplasia and follicular adenomas, the incidences of the latter being 65% and 87%, respectively in 1.5% and 3.0% KA-treated males, significantly higher than the control value of 2%. In the females, the figures were 2%, 8% and 80% in the 0%, 1.5% and 3.0% KA groups, respectively. The serum free T_3 levels in the 3.0% KA animals of both sexes at month 6 were significantly lower than in the controls. On the other hand, their serum TSH levels were higher, although the differences disappeared at later time points. In conclusion, continuous administration of high dose of KA induces thyroid adenomas in male and female B6C3F_1 mice, presumably by a mechanism involving decrease in serum free T_3 levels and increased TSH. © *1998 Elsevier Science Ltd. All rights reserved*

Abbreviation: KA = kojic acid.

Keywords: kojic acid; mice; thyroid tumorigenesis; TSH; T_3; T_4; antithyroid drug.

INTRODUCTION

Kojic acid (KA; 5-hydroxy-2-(hydroxymethyl)-4-pyrone) is produced by various strains of *Asperillus*, *Penicillum* and *Acetobactor* (Bajpai *et al.*, 1982; Parish *et al.*, 1966). It is therefore present in traditional Japanese foods such as miso, soy sauce and sake, albeit at very low concentrations (Sinshi *et al.*, 1984). In addition, KA is widely used as a food additive for preventing enzymatic browning, as well as for cosmetics.

KA has been subjected to mutagenicity testing and found to be a weak mutagen in bacteria but not in eukaryotic systems (Bjeldanes *et al.*, 1979; Kinoshita *et al.*, 1968; Shibuya *et al.*, 1982). Recently, however, it was established that KA can induce sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary cells in the presence or absence of a rat-liver S-9 mix (Wei *et al.*, 1991). Here we therefore investigated its potential tumorigenicity in (C57BL/6N × C3H/N)F_1 (B6C3F_1) mice.

MATERIALS AND METHODS

Animals and KA-containing diet

KA was obtained from Nagase Biochemical Company (Tokyo, Japan). It was combined with MF powdered basal diet (Oriental Yeast Company, Tokyo, Japan) at concentrations of 1.5 and 3.0% and then processed to pellets for convenience of handling. Totals of 195 male and 195 female B6C3F_1 mice were purchased from Charles River Co., Japan Inc. (Atsugi, Japan) at the age of 5 wk. They were maintained with free access to basal diet and tap water until the study was started at the age of 6 wk. They were housed six or seven to a plastic cage and kept in an air-conditioned room at 24 ± 2°C with relative humidity of 55 ± 5%. All animal experiments were carried out following the guidelines set by Hiroshima University in its 'Guide for the Care and Use of Laboratory Animals'.

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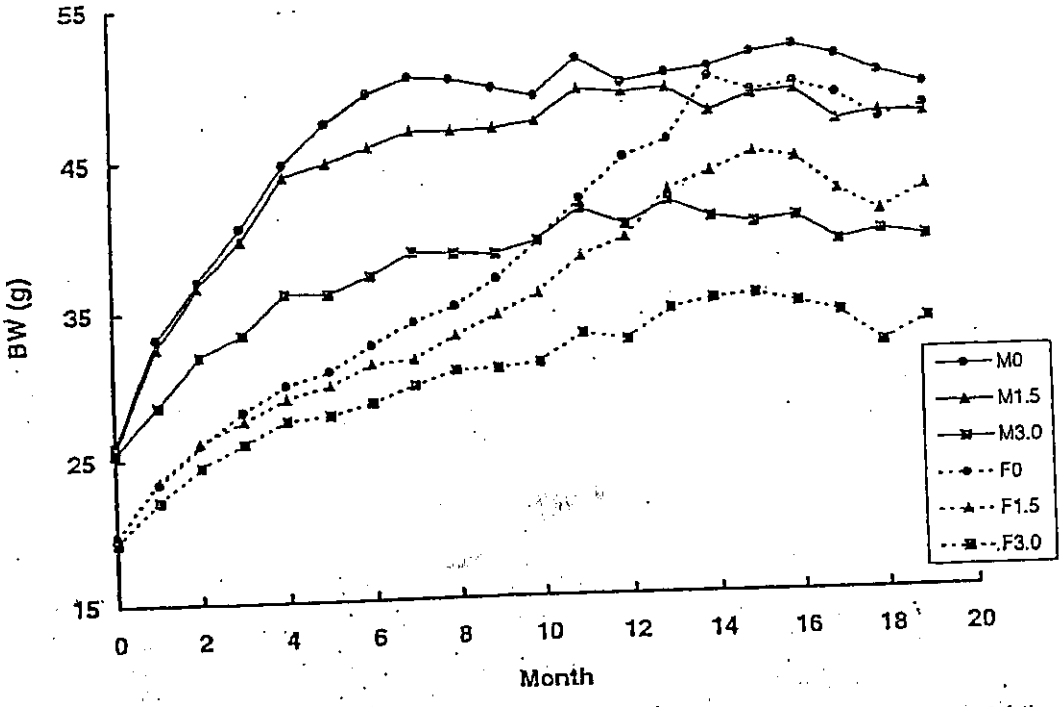

Fig. 1. Average body weight curves for B6C3F$_1$ mice treated with 0, 1.5 and 3% kojic acid in the diet.

Experimental design

Administration doses for the chronic toxicity test were chosen on the basis of preliminary subchronic (12 wk) test results for dose in the range of 0 to 5%. The mice were randomly divided into experimental groups, each consisting of 65 males and 65 females and given 0 (control), 1.5% and 3.0% KA-containing basal diet *ad lib*. Tap water was supplied *ad lib*. The animals were observed daily, and moribund animals were killed and autopsied along with those which died spontaneously. Body weights were noted every month. At 6 and 12 months after the commencement, subgroups of five animals were sacrificed and examined. At month 19, KA diet was switched to normal diet in 10–14 animals of each KA treated group. At the end of the 20 month (88 wk) experimental period, all surviving animals were sacrificed under ether anaesthesia and carefully examined. The thyroid, pituitary, liver, spleen, adrenals, kidneys, testes, ovaries and uterus were weighed. As thyroids are hard to dissect, they were weighed with a part of trachea attached. The same amount of the trachea was left so that the differences in weight could indicate thyroid weight changes. The organs above were fixed in 10% buffered formalin, routinely processed for wax embedding and sectioning. Sections were stained with haematoxylin eosin (HE) for histopathological assessment.

Table 1. Thyroid weights of BCF$_1$ mice treated with kojic acid

Group	Effective no. of mice	Thyroid weights (including part of the trachea)	
		Absolute (mg)	Relative (mg/10 g body weight)
M-0%	48	25 ± 1.4[a]	5.4 ± 0.31
M-1.5%	52	53 ± 3.1**	12 ± 0.70**
M-3.0%	53	71 ± 3.0**	19 ± 0.79**
F-0%	52	19 ± 0.8	4.2 ± 0.18
F-1.5%	51	24 ± 1.1**	6 ± 0.28**
F-3.0%	49	38 ± 3.3**	11 ± 0.97**

[a]Mean ± SEM.
**Significantly different from the control value at $P < 0.01$.

Hormone assay

Sera collected at months 6, 12 and 20, from five animals in each group were subjected to free T_3 and TSH assays. An Amarex-MAB free T_3 assay kit (Oso Clinical Diagnostic Co., Tokyo, Japan) was employed for free T_3 TSH concentrations were determined with a mouse TSH radioimmunoassay kit obtained from Dr A.F. Parlow at Pituitary Hormones and Antisera Centre, Harbor-UCLA medical centre (Torrance, CA, USA). The lactoperoxidase method was used for ^{125}I labelling of TSH (Thorell, 1971). The second antibody, anti guinea pig serum, was kindly provided by the Institute for Molecular and Cellular Regulation at Gunma University.

RESULTS

Survival rates, growth curves and diet consumption

Survival rates at 18 months (80 wk) were 67% in group M-0 (males on control diet), 56% in M-1.5 (males on 1.5% KA diet), 76% in M-3.0 (males, 3.0% KA), 91% in F-0 (females, control), 87% in F-1.5 (females, 1.5% KA) and 91% in F-3.0 (females, KA 3.0%) cases. Average body weight curves are shown in Fig. 1. Although increase in body weight was steady in all groups, the growth

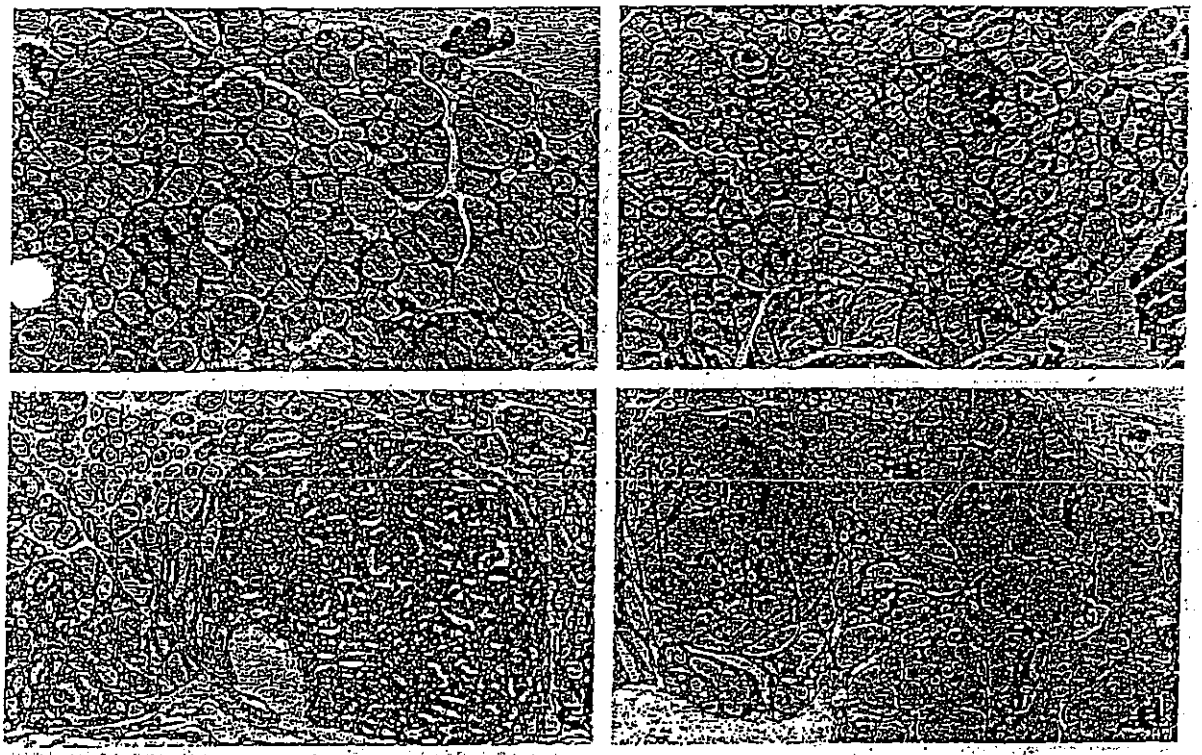

Plate 1. Thyroid lesions in $B6C3F_1$ mice treated with kojic acid (KA) in the diet for 20 months: (a) Control thyroid of a male mouse (HE staining, ×100); (b) hyperplasia in a male mouse receiving 1.5% KA (HE, ×40); (c) follicular adenoma within an area of hyperplasia in a male mouse given 3.0%KA (HE, ×40); (d) follicular adenoma with a large amount of colloid in a male mouse receiving 3.0% KA (HE, ×40).

Table 2. Incidence of tumours in BCF_1 mice treated with kojic acid

Group	Effective no. of mice	Thyroid adenoma[a]	Hepatoma	Lymphoma	Other tumours
M-0%	48	1 (2%)	23 (48%)	0 (0%)	4 (Harderian g.t., 3; pulmonary t., 1)
M-1.5%	52	34 (65%)**	36 (69%)	0 (0%)	2 (skin t., 2)
M-3.0%	53	46 (87%)**	25 (47%)	1 (2%)	3 (skin t., 1; Harderian g.t., 1; gastric t., 1)
F-0%	52	1 (2%)	0 (0%)	1 (2%)	3 (mammary t., 2 uterus t., 1; pulmonary t., 1)
F-1.5%	51	4 (8%)	2 (4%)	5 (10%)	4 (Peyer p.t., 2; mammary t., 2)
F-3.0%	49	39 (80%)**	5 (10%)*	1 (2%)	4 (Harderian g.t., 2; uterus t., 1; mammary t., 1)

*,**Significantly different from the control value at $P < .05$ or $P < .01$, respectively.
[a]Incidences include "KA interrupted" animals (see Materials and Methods). The incidences excluding "KA interrupted" were 27/40 (68%) in M-1.5, 40/43 (93%) in M-3.0, 2/37 (5%) in F-1.5 and 30/37 (81%) in F-3.0, also showing the same significant differences from the controls.

Table 3. Modification of thyroid adenoma incidence by withdrawal of kojic acid administration

		Uninterrupted		Interrupted		
Group	Thyroid wt[a] (mg)	Hyperplasia[b]	Adenoma[b]	Thyroid wt (mg)	Hyperplasia	Adenoma
M-1.5%	42 ± 8.7	0/13 (0)	13/13 (100%)	46 ± 24.6	5/12 (42%)**	7/12 (58%)**
M-3.0%	69 ± 20.1	2/29 (7%)	27/29 (93%)	57 ± 28.5	4/10 (40%)*	6/10 (60%)*
F-1.5%	22 ± 3.4	3/25 (12%)	2/25 (8%)	24 ± 7.7	4/14 (29%)	2/14 (14%)
F-3.0%	37 ± 15.3	4/26 (15%)	21/26 (81%)	39 ± 34.8	3/12 (25%)	9/12 (75%)

*Significantly different from the control value at $P < .05$ or $P < .01$, respectively.
[a]Thyroids were weighed with part of the trachea attached.
[b]All of the hyperplasia observed were diffused types. Adenomas were follicular type.

rates were significantly suppressed in groups M-3.0, F-1.5 and F-3.0. Diet consumption, however, did not decrease in the mice receiving KA, except for an initial drop in the highest dose groups (data not shown).

Final organ weights and blood analysis

The thyroid, pituitary, liver, spleen, adrenal, kidneys, testes, ovaries and uterus were weighed at autopsy. Thyroid weight were increased significantly in the KA-fed groups of both sexes (Table 1), especially in the male groups. Except for the thyroid, there were no significant differences among groups in the major organ weights or values for haematological and serum biochemical parameters, including white blood cell counts, red blood cell counts, albumin, glutamic–oxaloacetic transaminase (GOT), chorine esterase (ChE), lactate dehydrogenase (LDH), leucine amino peptide (LAP) and total cholesterol (T-CHO).

Tumours

Table 2 summarizes data for the incidences of tumours in various organs. In the KA-treated groups, thyroid tumours were the most common lesions. Histologically they were classified as hyperplasia and follicular adenomas (Plate 1). All of the hyperplasia observed were diffused type composed of follicles with irregular shapes containing large amount of colloid and lined by tall epithelial cells. Adenomas showed uniform cell growth with complete collapse of the normal follicular structure. However, small amounts of colloid (on rare occasions, large amounts) were seen in adenomas. In all male groups, the incidences of hepatomas were high (47–69%), without any significant intergroup variation. In females, on the other hand, the value of 10% in the F-3.0 group was significantly elevated as compared with the control 0%. Lymphomas, mammary tumours, Harderian gland, skin, Peyer plate, uterine and gastric tumours were also observed at relatively low incidences and none showed any correlation with the KA treatment.

Effect of interruption of KA diet on thyroid tumours

KA diet was switched to normal diet in 10–14 animals of each group 30 days before the termination. In those interrupted animals, incidences of thyroid adenomas significantly decreased, although

Table 4. Serum free T_3 in BCF_1 mice treated with kojic acid

Group	Month 6	Month 12	Month 20
M-0%	3.65 ± 0.375[a]	2.22 ± 0.219	4.17 ± 0.381
M-1.5%	3.67 ± 0.428	2.84 ± 0.186	4.23 ± 0.330
M-3.0%	1.56 ± 0.334**	1.40 ± 0.136*	3.69 ± 0.214*
F-0%	3.32 ± 0.189	2.75 ± 0.306	3.39 ± 0.352
F-1.5%	1.42 ± 0.339**	2.67 ± 0.225	2.51 ± 0.225**
F-3.0%	1.45 ± 0.138**	2.83 ± 0.295	2.71 ± 0.274**

[a]Mean ± SEM (pg/ml, n = 5).
*,**Significantly different from the control value at $P < 0.05$ or $P < 0.01$, respectively.

Table 5. Serum TSH levels in BCF_1 mice treated with kojic acid

Group	Month 6	Month 12	Month 20
M-0%	30.6 ± 8.25[a]	22.5 ± 1.84	21.9 ± 2.07
M-1.5%	39.3 ± 3.98	25.4 ± 0.98	17.7 ± 1.78
M-3.0%	53.9 ± 6.52	23.3 ± 1.97	16.0 ± 0.39**
F-0%	23.3 ± 2.45	16.0 ± 0.78	13.7 ± 1.68
F-1.5%	38.8 ± 1.22**	16.9 ± 1.97	13.9 ± 0.75
F-3.0%	36.3 ± 5.73	18.5 ± 1.03	15.1 ± 0.91

[a]Mean ± SEM (pg/ml, n = 5).
*,**Significantly different from the control value at $P < 0.01$.

the average thyroid weights were unchanged (Table 3). However, in females, no significant effects were noted.

Serum free T_3 and TSH

Data for serum free T_3 and TSH levels at months 6, 12 and 20 are summarized in Tables 4 and 5, respectively. At month 6, free T_3 levels were significantly decreased in the M-3.0, F-1.5 and F-3.0 mice, while TSH levels were increased. Free T_3 levels continued to be decreased in some groups, thereafter but no consistent change was evident for TSH.

DISCUSSION

KA is widely used as a food additive as well as in the manufacture of cosmetics. As it was recently found to induce sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary cells (Wei *et al.*, 1991), an examination of its carcinogenicity in animals was clearly necessary. The present study demonstrated that continuous oral administration of KA can induce thyroid adenomas in both sexes of mice. No thyroid carcinomas were noted despite an adenoma incidence of 87% in the M-3.0 group. Among the other tumours, the incidence in hepatocellular adenomas in the F-3.0 mice was also significantly greater than that in the control.

Although the growth rates were suppressed with the doses of KA chosen in the present study, all animals remained in good health throughout the experiment and survival rates did not differ among the groups. Enlargement of the thyroids was evident in males at month 12, when five animals of each group were sacrificed. At this time point, all mice in group M-3.0 and two of five in M-1.5 were found to have adenomas. However, the nature of these lesions is clearly dependent to some extent an continuous simulation since considerable reversibility was evident on comparison of the interrupted and non-interrupted groups.

It is unlikely that induction of thyroid tumours by KA is due to genotoxicity, considering that almost the entire thyroids were hyperplastic in the M-1.5, M-3.0 and F-3.0 mice. It is well known in rodents that antithyroid drugs (goitrogenic substances) or a low iodine diet cause hyperplasia in the thyroid and that tumours develop in the long term (Biancifiori, 1979; Hill *et al.*, 1989; McClain, 1989; Morris, 1955). These substances interfere with synthesis and/or metabolism of thyroid hormones and eventually decrease serum thyroid hormone levels (Nakashima *et al.*, 1978; Tauro and Dorris, 1989). It is also possible that KA increased hepatic metabolism and excretion of thyroid hormones. Various drugs such as phenobarbital and amiodarone have known to facilitate T_3 excretion in the liver and reduce serum thyroid hormones. In either case, the pituitary gland produces more TSH in turn and this stimulates follicular cell hypertrophy. Thus, from the present results KA appears to act as an antithyroid substance, partly evidenced by the findings for T_3 and TSH. The differences in growth curves between groups were also consistent with decreases in serum free T_3. In severe hypothyroid conditions such as chronic iodine deficiency, TSH cells in the pituitary gland are known to proliferate and form TSH cell clusters (Furth *et al.*, 1973). In the present study, however, the anterior pituitary did not show any pathological changes in the KA groups (data not shown), in line with the normal level of TSH which appeared in KA-treated groups in the later point of the experiment. On the other hand, thyroid tumour development appeared to continue throughout so that discrepancy between two parameters requires explanation.

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