Jpn. J. Cancer Res. 83, 1279–1285, December 1992

Forestomach Neoplasm Induction in F344/DuCrj Rats and B6C3F$_1$ Mice Exposed to Sesamol

Seiko Tamano, Masao Hirose, Hikaru Tanaka, Emiko Asakawa, Kumiko Ogawa and Nobuyuki Ito

First Department of Pathology, Nagoya City University Medical School, 1-Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467

Sesamol was administered at a dietary level of 2% to groups of 30 male and female F344/DuCrj rats and B6C3F$_1$ mice for 104 and 96 weeks, respectively. Squamous cell carcinomas in the forestomach were induced in nine of 29 (31%) effective male rats, three of 30 (10%) female rats, eleven of 29 (38%) male mice and five of 30 (17%) female mice treated with sesamol. Papillomas developed in ten of 29 (34%) male rats and fourteen of 30 (47%) female rats, but not in any of the mice. Hyperplasias developed in almost all rats and mice of both sexes. Significant differences from control values were found for all three lesions in rats and for carcinoma and hyperplasia categories in mice. The incidences of other tumors in the 2% sesamol group were comparable with control values. In conclusion, sesamol induces squamous cell carcinomas in the forestomach of rats and mice, males being more susceptible than females.

Key words: Sesamol — Antioxidant — F344 rat — B6C3F$_1$ mouse — Carcinogenicity

Synthetic and naturally occurring phenolic antioxidants are widely used as food additives and are therefore present in our environment at high levels. They are generally not mutagenic as evaluated in several mutagenesis assay systems,[1] and may even indirectly decrease the mutagenic activity of known mutagens.[2,3] Moreover, they inhibit chemical carcinogenesis in various organs of rats or mice when they are given prior to and/or simultaneously with certain carcinogens.[4] However, since Ito *et al.* first found that butylated hydroxyanisole (BHA) can induce forestomach carcinomas in rats of both sexes,[5] a number of structurally related phenolic compounds have been found to have possible carcinogenic potential.[6–8]

Sesamol, one of the naturally occurring antioxidants which is a minor component of sesame seed oil,[9] was recently shown to induce strong cell proliferation in rat forestomach epithelium in a short-term experiment,[10] as well as papillomas in the same organ of rats in a 60-week experiment.[8,11] These results strongly suggested that sesamol is carcinogenic for rat forestomach epithelium. The present long-term experiment was performed using F344/DuCrj (F344) rats and B6C3F$_1$ mice of both sexes to establish whether the rodent forestomach is susceptible to sesamol carcinogenicity.

MATERIALS AND METHODS

Test chemical Sesamol (3,4-methylenedioxyphenol, CAS No. 533-31-3, purity >98%) was purchased from Fluka Chemie, AG. (Switzerland).

Animals and maintenance Male and female F344 rats and B6C3F$_1$ mice, aged 5 weeks, were purchased from Charles River Japan Inc., Atsugi and quarantined for 7 days before starting the experiment. Animals of the same sex were housed five to a polycarbonate cage with hard wood Beta Chips (Northeastern Product Co., Warrensburg, NY) for bedding. The animals were supplied with feed (Oriental MF powdered diet, Oriental Yeast Co., Ltd., Tokyo) and tap water *ad libitum*. Bedding and cages were changed 2 times a week. The room temperature and relative humidity were controlled at 22±2°C and 60±10% and the room air was changed more than 15 times per hour. Fluorescent lighting was adjusted to provide a 12-h light/dark cycle. Animals were weighed and randomly assigned to dose groups the day before starting the study.

Diet preparation Diets containing 2% sesamol were prepared by mixing weighed quantities of the antioxidant and Oriental MF powdered diet in a stainless-steel mixer for 30 min. Diet was prepared every 4 weeks and stored in a freezer (−22 ~ −26°C) until use. Analysis of a sample of blended diet showed that the actual level of sesamol in the mixture was 1.94% (analyzed by Japan Food Research Laboratories). Sesamol in the diet was confirmed to be stable over 4 weeks (the actual level of sesamol in mixture containing 2% was 1.88% after storage in a freezer for 4 weeks).

Experimental procedure Groups of 30 rats and mice of each sex were given diets containing 0 (control) or 2% sesamol for 104 and 96 weeks, respectively. The animals were observed twice daily for abnormalities. Individual body weights were recorded weekly for the first 14 weeks and then every 4 weeks. Food and water consumptions were measured for 2 days before each weighing. In week

96, fresh urine samples were obtained from 5 mice in each group and their pH, protein, glucose, ketones, bilirubin, occult blood and urobilinogen contents were measured with Multistix and specific gravity by using a refractometer (Atago Co. Ltd., Tokyo). At the end of the treatment period, surviving animals were deprived of food, but not water, overnight and then killed under ether anesthesia by exsanguination from the abdominal aorta. Hematological examinations of blood samples included an erythrocyte count, a leukocyte count, measurements of the hemoglobin concentration and hematocrit value and a platelet count (Sysmex microcell counter CC-180A, Towa Iryo Denshi Co., Ltd., Tokyo). The serum levels of glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), alkaline phosphatase, total cholesterol, total protein, the albumin:globulin ratio, urea nitrogen, glucose and albumin were measured. Gross examination was performed at autopsy, and detailed inspections of the luminal surfaces of the intestine and urinary bladder were also carried out after fixation. The following organs were weighed and the organ-to-body weight ratios were determined: the liver and kidneys for rats and the brain, heart, liver, spleen, kidneys and testes or ovaries for mice. Samples of these organs and of the salivary glands, trachea, lungs, thymus, lymph nodes, stomach, small intestine, large intestine, pancreas, urinary bladder, pituitary, thyroid, adrenals, prostate, seminal vesicle, skin, mammary gland, skeletal muscle, spinal cord, sciatic nerve and any other tissues with abnormal appearance were fixed in 10% buffered formalin. For microscopic examination, tissues were embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin. Histopathological examinations were also performed on animals that died or were killed when they became moribund during the experiment.

Statistical analyses Tumor incidences were analyzed by using the one-sided Fisher's exact probability test. Other data were analyzed by using Student's *t* test.

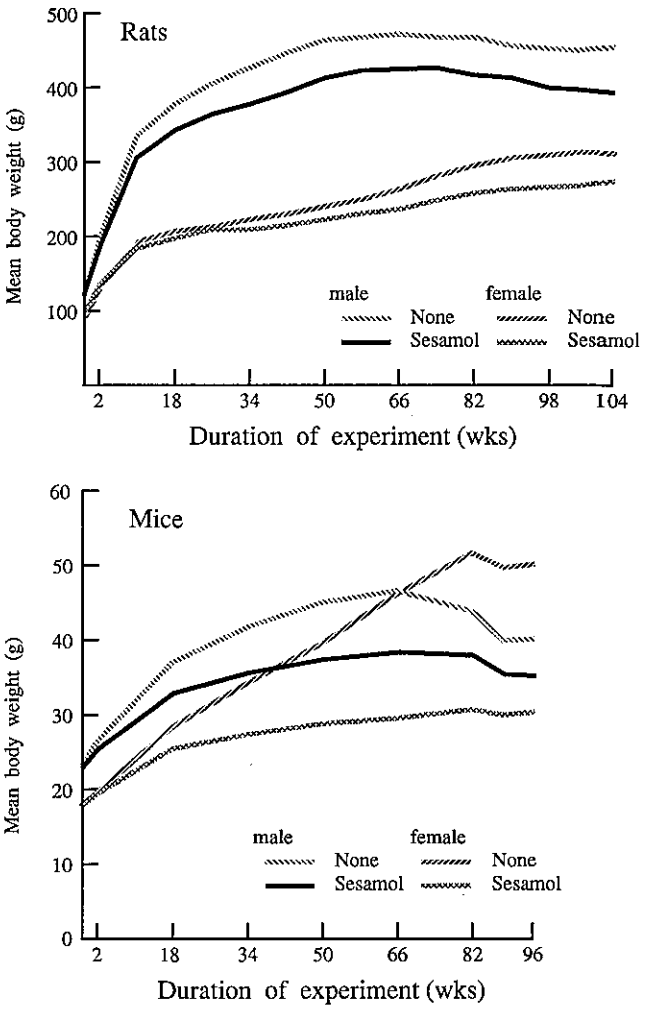

Fig. 1. Growth curves of male and female F344 rats and B6C3F$_1$ mice maintained on diets containing sesamol at concentrations of 0 and 2.0%.

RESULTS

Rats No clinical signs which could be related to sesamol treatment were apparent in any of the rats during the 104 week experiment. At the termination, the survival rates of males and females fed 0 and 2.0% sesamol were 80 and 90, and 73.3 and 86.7%, respectively. There was no significant difference between the mortalities of controls and sesamol-treated rats at any time during the 104 week experiment.

The mean body weights of male and female rats given 2.0% sesamol were consistently less than those of controls from week 1 to the termination (Fig. 1). The food consumption and water intake of controls and sesamol-treated animals of both sexes were not different. The relative liver and kidney weights were lower than control values in both sexes. Gross observation revealed white/gray nodules in the forestomach of both sexes treated with sesamol.

Incidences of tumors developing in sesamol-treated F344 rats are summarized in Table I. Hyperplasias, papillomas and squamous cell carcinomas (SCCs) in the forestomach were evident in both sexes fed a diet of 2.0% sesamol for 104 weeks. These changes in the forestomach were classified as previously reported.[12, 13] The incidence of SCC in males was higher than that in females, but this was not statistically significant. SCCs were mostly of well to moderately differentiated type (Fig. 2) except for one poorly differentiated lesion found in a male rat.

Table I. Incidences of Hyperplastic Lesions and Tumors Developing in Sesamol-treated F344 Rats

Site and type of tumor	Males		Females	
	0%	2.0%	0%	2.0%
Effective no. of rats	30	29	30	30
Thymus				
Thymoma	0	0	0	1 (3)
Pituitary				
Adenoma, pars distalis	2 (7)	0	5 (17)	11 (37)
Carcinoma	0	0	2 (7)	0
Thyroid				
Follicular cell adenoma	0	0	1 (3)	0
C-cell adenoma	3 (10)	3 (10)	1 (3)	1 (3)
C-cell carcinoma	1 (3)	0	1 (3)	0
Adrenal				
Pheochromocytoma	4 (13)	0	0	0
Malignant pheochromocytoma	1 (3)	0	0	0
Lung				
Adenoma	2 (7)	0	0	1 (3)
Tongue				
Papilloma	0	1 (3)	0	1 (3)
Carcinoma	1 (3)	1 (3)	0	0
Forestomach				
Hyperplasia	1 (3)	29 (100)**	5 (17)	30 (100)**
Papilloma	0	10 (34)**	0	14 (47)**
Squamous cell carcinoma	0	9 (31)**	0	3 (10)
Glandular stomach				
Submucosal hyperplasia	0	0	0	1 (3)
Adenoma	0	1 (3)	0	0
Large intestine				
Adenoma	0	1 (3)	0	0
Pancreas				
Acinar cell adenoma	1 (3)	0	0	0
Islet-cell adenoma	0	0	1 (3)	0
Liver				
Hyperplastic nodule	2 (7)	0	2 (7)	0
Hepatocellular carcinoma	1 (3)	0	0	0
Testes				
Interstitial cell tumor	29 (97)	29 (100)	–	–
Prostate				
Adenoma	3 (10)	1 (3)	–	–
Mammary gland				
Adenoma	–	–	2 (7)	0
Fibroadenoma	–	–	4 (13)	2 (7)
Adenocarcinoma	–	–	0	1 (3)
Uterus				
Adenocarcinoma	–	–	0	1 (3)
Endometrial stromal polyp	–	–	8 (27)	8 (27)
Endometrial stromal sarcoma	–	–	0	1 (3)
Preputial/clitoral gland				
Adenoma	1 (3)	0	0	1 (3)
Skin/subcutis				
Trichoepithelioma	1 (3)	0	0	0
Fibroma	1 (3)	0	0	0
Lipoma	1 (3)	1 (3)	0	0
Myxoma	0	1 (3)	0	0
Zymbal's gland				
Squamous cell carcinoma	0	0	1 (3)	0
Abdominal cavity				
Mesothelioma	1 (3)	0	0	0
All sites				
Malignant lymphoma/leukemia	5 (17)	0	6 (20)	1 (3)

**: $P<0.01$.

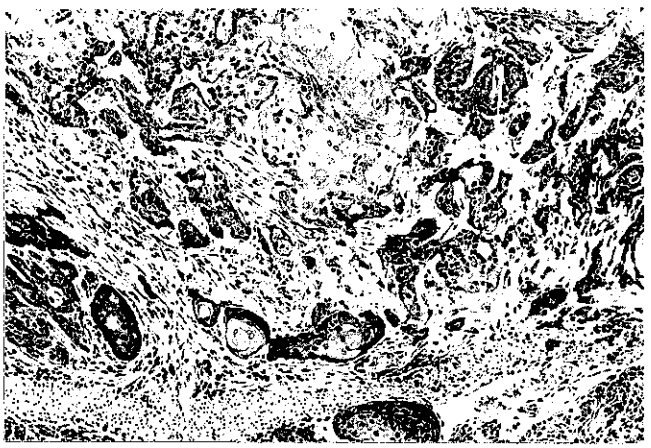

Fig. 2. Moderately differentiated squamous cell carcinoma in a male F344 rat given 2.0% sesamol. Small spindle-shaped squamous cells are observed invading the muscle layer. Keratinized well-differentiated squamous cells are present in the upper central region. H-E, ×110.

The incidences of tumors developing in organs other than the forestomach were comparable with those of controls for both sexes. No obvious toxic changes due to sesamol treatment were found in any organ.

Mice No clinical signs which could be related to sesamol treatment were apparent in any of the mice during the 96 week treatment. The survival rates of males and females fed 0 and 2.0% sesamol were very similar at 76.7 and

Table II. Incidences of Hyperplastic Lesions and Tumors Developing in Sesamol-treated $B6C3F_1$ Mice

Site and type of tumor	Males		Females	
	0%	2.0%	0%	2.0%
Effective no. of mice	28	29	29	30
Spleen				
Hemangiosarcoma	1 (4)	1 (3)	0	0
Pituitary				
Adenoma, pars distalis	0	0	0	1 (3)
Carcinoma	1 (4)	0	1 (3)	0
Lung				
Adenoma	0	1 (3)	0	0
Adenocarcinoma	0	0	1 (3)	1 (3)
Tongue				
Papilloma	1 (4)	0	0	0
Forestomach				
Hyperplasia	1 (4)	29 (100)**	3 (10)	28 (93)**
Squamous cell carcinoma	0	11 (38)**	0	5 (17)*
Sarcoma	0	0	0	1 (3)
Glandular stomach				
Submucosal hyperplasia	0	0	0	5 (17)*
Sarcoma	0	1 (3)	0	0
Liver				
Hyperplastic nodule	7 (25)	5 (17)	0	1 (3)
Hepatocellular carcinoma	5 (19)	2 (7)	1 (3)	0
Hemangioma	3 (11)	0	0	0
Hemangiosarcoma	1 (4)	0	0	0
Mammary gland				
Adenocarcinoma	0	0	0	1 (3)
Uterus				
Endometrial stromal polyp	–	–	0	2 (7)
Endometrial stromal sarcoma	–	–	0	1 (3)
Skin/subcutis				
Fibrosarcoma	1 (4)	0	0	0
Harderian gland				
Adenoma	0	1 (3)	3 (10)	0
Adenocarcinoma	0	1 (3)	0	0
All sites				
Malignant lymphoma/leukemia	2 (7)	3 (10)	3 (10)	5 (17)

*, **: $P<0.05$, 0.01.

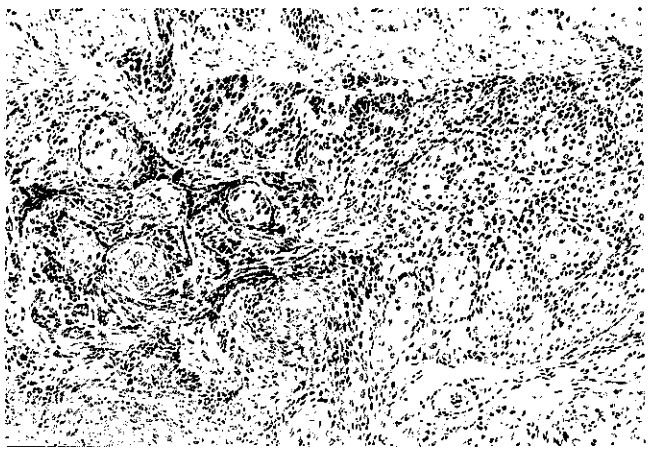

Fig. 3. Moderately differentiated squamous cell carcinoma in a male B6C3F$_1$ mouse given 2.0% sesamol. Small spindle-shaped squamous cells demonstrate massive invasion into the muscle layer. H-E, ×135.

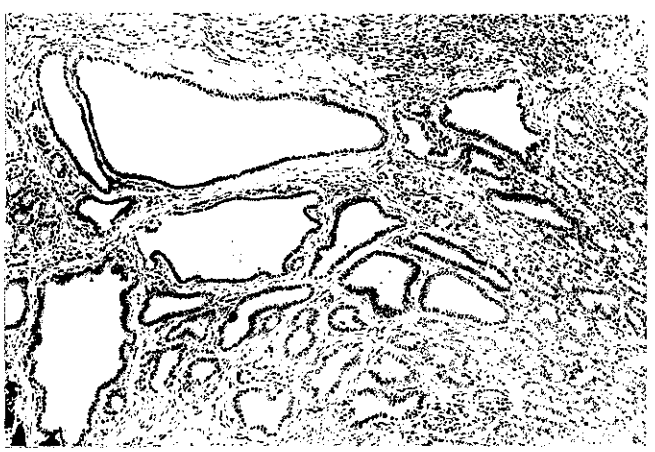

Fig. 4. Submucosal hyperplasia in a female B6C3F$_1$ mouse given 2.0% sesamol. Irregularly dilated glands lined by a single layer of epithelial cells are observed in the submucosa with accompanying fibrosis. H-E, ×170.

63.3% and 86.7 and 73.3%, respectively. Body weights of treated male and female mice were significantly decreased in comparison with the controls from week 1 and week 8 to the termination, respectively (Fig. 1). The food consumption and water intake of controls and sesamol-treated animals of both sexes were not different.

Urine analyses did not reveal any marked changes related to sesamol treatment. Erythrocyte count values were slightly decreased in females given 2.0% sesamol. In the serum chemistry data, GOT, alkaline phosphatase and blood urea nitrogen values were slightly increased, and serum glucose and total protein values were slightly decreased in females given 2.0% sesamol. There were no such differences between control and sesamol-treated males (data not shown). Statistically significant increases in the relative weights of the brain, heart, liver, spleen and kidneys in females, and the brain in males given 2.0% sesamol were observed, which seemed to be related to the retardation of body weight increase (data not shown).

On gross observation, raised or nodular surfaces were observed in the forestomach of both sexes treated with sesamol.

Incidences of tumors developing in sesamol-treated B6C3F$_1$ mice are summarized in Table II. In both sexes significant induction of hyperplasias and SCCs, which were mostly of well to moderately differentiated type (Fig. 3) except for one poorly differentiated lesion in a male mouse was noted, but no papillomas. In the glandular stomach, a significant incidence of submucosal hyperplasia was present in female mice (Fig. 4). Single sarcomas of the forestomach and glandular stomach were respectively found in one female and one male receiving 2% sesamol. The incidences of tumors developing in organs other than the forestomach were comparable with those of controls of both sexes.

DISCUSSION

The present study of the sesamol carcinogenicity in rats and mice of both sexes using a dietary concentration of 2.0% revealed selective development of SCCs in the forestomach of these rodents, associated with hyperplasia. Therefore, the present results were considered to provide sufficient evidence of carcinogenicity. No toxicological effects were demonstrated other than in the forestomach epithelium and no clinical signs or alterations in laboratory analysis parameters related to sesamol treatment were observed in mice or rats except for retardation of body weight increase.

Many antioxidants, including sesamol, BHA, caffeic acid, 2-*tert*-butyl-4-methylphenol, 4-*tert*-butylphenol, 4-methoxyphenol and methylhydroquinone, have been reported to elicit proliferative responses in the forestomach epithelium of rats and hamsters.[6, 10] Of these chemicals, BHA, caffeic acid, sesamol and 4-methoxyphenol induce severe hyperplasia, with BHA and caffeic acid eventually being proven to be complete forestomach carcinogens.[5, 12, 14, 15] Therefore, carcinogenicity for sesamol and 4-methoxyphenol has been strongly suspected. Indeed, forestomach carcinogenicity has recently been demonstrated for 4-methoxyphenol in a 2-year feeding study in F344 male and female rats.[16] Therefore, strong cell proliferation appears to be directly correlated with carcino-

genic activity, particularly in the case of such so-called non-genotoxic carcinogens. Early forestomach changes induced by 2% sesamol are characterized by an increase in DNA synthesis at 3 days after treatment followed by hyperplasia, epithelial necrosis, ulceration and inflammation (unpublished observations). At a dose of 1%, although an increase in DNA synthesis was apparent, hyperplasia and ulceration were not found.[10] Therefore sesamol or its metabolite(s) primarily induce cell proliferation at high dose with chronic epithelial damage stimulating continued regeneration.

Recently, however, small incidences of irreversible dysplastic foci were found in animals treated with 2% sesamol or caffeic acid for 24 weeks followed by a return to basal diet alone for a further 24 weeks.[17] This indicates persistent lesions normally associated with heritable genetic alterations and in fact, weak spots indicating the presence of DNA-adducts were demonstrated in the forestomach of rats treated with 2% BHA for 2 weeks.[18] Similar dysplastic foci were observed much more frequently in animals treated with the genotoxic agents N-methyl-N′-nitro-N-nitrosoguanidine or N-methylnitrosourethane for 24 weeks alone or for 24 weeks followed by basal diet for an additional 24 weeks.[11] Therefore, it is likely that during the continuous strong cell proliferation occurring in the present study, DNA alteration could have occurred in the epithelial cells, leading to conversion of hyperplasias into dysplasias or neoplasias. It is of interest that both sesamol and 4-methoxyphenol produced similar circular ulcers in the mid region of the forestomach in addition to hyperplasia.[6, 10, 12, 19] Whether similar metabolite(s) or mechanism(s) to those involved in the generation of toxicity and cell proliferation also result in the carcinogenicity requires clarification. Since female mice developed submucosal hyperplasia in the glandular stomach, this might indicate that the forestomach epithelium is not the only site susceptible to sesamol.

With regard to risk assessment in man, sesamol can still be considered a low risk compound, because humans do not possess a forestomach and because of the high threshold level, similar to that found for the synthetic phenolic antioxidant BHA.[5, 20] Human ingestion is assumed to be at quite low levels, because sesamol is only found in sesame seed oil at levels of 0.004 to 0.05%, depending on the processing method.

In conclusion, the present study demonstrated that 2% sesamol in the diet can exert forestomach carcinogenic activity in F344 rats and $B6C3F_1$ mice with the incidence of lesions in males being higher than in females. In addition, limited glandular stomach carcinogenic potential in female mice was suggested.

ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan, as well as grants from the Experimental Pathology Research Association, Japan, and the Society for Promotion of Pathology of Nagoya, Japan.

(Received July 13, 1992/Accepted September 17, 1992)

REFERENCES

1) Boninn, A. M. and Baker, R. S. U. Mutagenicity testing of some approved food additives with the *Salmonella*/microsome assay. *Food Technol. Aust.*, **32**, 608–611 (1980).
2) Calle, L. M. and Sullivan, P. D. Screening of antioxidants and other compounds for antimutagenic properties toward benzo(a)pyrene-induced mutagenicity in strain TA 98 of *Salmonella typhimurium*. *Mutat. Res.*, **101**, 99–114 (1982).
3) Kahl, R. Synthetic antioxidants: biochemical actions and interference with radiation, toxic compounds, chemical mutagens and chemical carcinogens. *Toxicology*, **33**, 185–228 (1984).
4) Wattenberg, L. W. Chemoprevention of cancer. *Cancer Res.*, **45**, 1–8 (1985).
5) Ito, N., Fukushima, S., Hagiwara, A., Shibata, M. and Ogiso, T. Carcinogenicity of butylated hydroxyanisole in F344 rats. *J. Natl. Cancer Inst.*, **70**, 343–352 (1983).
6) Hirose, M., Inoue, T., Asamoto, M., Tagawa, Y. and Ito, N. Comparison of the effects of 13 phenolic compounds in induction of proliferative lesions of the forestomach and increase in the labelling indices of the glandular stomach and urinary bladder epithelium of Syrian golden hamsters. *Carcinogenesis*, **7**, 1285–1289 (1986).
7) Ito, N. and Hirose, M. The role of antioxidants in chemical carcinogenesis. *Jpn. J. Cancer Res.*, **78**, 1011–1026 (1987).
8) Ito, N. and Hirose, M. Antioxidants — carcinogenic and chemopreventive properties. *Adv. Cancer Res.*, **53**, 247–302 (1989).
9) Kikugawa, K., Kunugi, A. and Kurechi, T. Chemistry and implications of degradation of phenolic antioxidants. *In* "Food Antioxidants," ed. B. J. F. Hudson, pp. 65–98 (1991). Elsevier Applied Science, London and New York.
10) Hirose, M., Masuda, A., Imaida, K., Kagawa, M., Tsuda, H. and Ito, N. Induction of forestomach lesions in rats by oral administrations of naturally occurring antioxidants for 4 weeks. *Jpn. J. Cancer Res.*, **78**, 317–321 (1987).
11) Ito, N., Hirose, M., Hagiwara, A. and Takahashi, S. Carcinogenicity and modification of carcinogenic response

by antioxidants. *In* "Antimutagenesis and Anticarcinogenesis Mechanisms II," ed. Y. Kuroda, D. M. Shankel and M. D. Waters, pp. 183–194 (1990). Plenum Publishing Corporation, New York.

12) Hirose, M., Fukushima, S., Kurata, Y., Tsuda, H., Tatematsu, M. and Ito, N. Modification of N-methyl-N′-nitro-N-nitrosoguanidine-induced forestomach and glandular stomach carcinogenesis by phenolic antioxidants in rats. *Cancer Res.*, **48**, 5310–5315 (1988).

13) Hirose, M., Yamaguchi, S., Fukushima, S., Hasegawa, R., Takahashi, S. and Ito, N. Promotion by dihydroxybenzene derivatives of N-methyl-N′-nitro-N-nitrosoguanidine-induced F344 rat forestomach and glandular stomach carcinogenesis. *Cancer Res.*, **49**, 5143–5147 (1989).

14) Hagiwara, A., Hirose, M., Takahashi, S. Ogawa, K., Shirai, T. and Ito, N. Forestomach and kidney carcinogenicity of caffeic acid in F344 rats and C57BL/6N× C3H/HeN F_1 mice. *Cancer Res.*, **51**, 5655–5660 (1991).

15) Hirose, M., Fukushima, S., Shirai, T., Hasegawa, R., Kato, T., Tanaka, H., Asakawa, E. and Ito, N. Stomach carcinogenicity of caffeic acid, sesamol and catechol in rats and mice. *Jpn. J. Cancer Res.*, **81**, 207–212 (1990).

16) Asakawa, E., Hirose, M., Imaida, K., Hagiwara, A., Uwagawa, S. and Ito, N. Carcinogenicity of 4-methoxyphenol and 4-methylcatechol in F344 rats. *Proc. Jpn. Cancer Assoc., 51st Annu. Meet.*, 52 (1992) (in Japanese).

17) Hirose, M., Masuda, A., Hasegawa, R., Wada, S. and Ito, N. Regression of butylated hydroxyanisole (BHA)-induced hyperplasia but not dysplasia in the forestomach of hamsters. *Carcinogenesis*, **11**, 239–244 (1990).

18) Nakagawa, S., Kogiso, S., Yoshitake, A., Hirose, M. and Ito, N. Effects of sodium nitrite on the DNA adduct formation in the stomach of catechol- or BHA-treated rats. *Proc. Jpn. Cancer Assoc., 50th Annu. Meet.*, 45 (1991) (in Japanese).

19) Rodrigues, C., Lok, E., Nera, E., Iverson, F., Page, D., Karpinski, K. and Clayson, D. B. Short-term effects of various phenols and acids on the Fischer 344 male rat forestomach epithelium. *Toxicology*, **38**, 103–117 (1986).

20) Ito, N., Fukushima, S., Tamano, S., Hirose, M. and Hagiwara, A. Dose response in butylated hydroxyanisole induction of forestomach carcinogenesis in F344 rats. *J. Natl. Cancer Inst.*, 77, 1261–1265 (1986).