Jpn. J. Cancer Res. 81, 738–741, August 1990

Induction of Hepatic Tumors with Butylated Hydroxyanisole in the Self-fertilizing Hermaphroditic Fish *Rivulus ocellatus marmoratus*

Eun-Ho Park,[1,3] Hwa-Hyoung Chang[1] and Young-Nam Cha[2]

[1]*Department of Biology, College of Natural Sciences, Hanyang University, Seoul 133-791 and* [2]*Department of Pharmacology and Toxicology, School of Medicine, Inha University, Inchon 402-751, Korea*

The three-day-old larvae of self-fertilizing hermaphroditic fish *Rivulus ocellatus marmoratus* were fed a diet containing the antioxidant butylated hydroxyanisole (BHA) at levels of 0.01–0.8% for 12 days. Six months after BHA administration, hepatic tumors were found in all groups of BHA-treated fish. The BHA-induced tumor incidences were clearly dose-dependent. These results show that dietary BHA is hepatocarcinogenic in *Rivulus* even at 0.01% dose.

Key words: Hepatic tumor induction — Butylated hydroxyanisole — Hermaphroditic fish

The antioxidant butylated hydroxyanisole (BHA) is used widely as a chemical preservative in various lipid-containing processed foods and in many cosmetic preparations. This compound is absorbed easily from the gastrointestinal tract and is extensively metabolized in the liver of both animals and humans.[1-3] BHA itself failed to show genotoxicity in a series of *in vivo* and *in vitro* assays,[4] and this compound is known to inhibit the mutagenic[5] and carcinogenic effects[6,7] of a broad range of chemical carcinogens. Earlier studies with BHA did not reveal any carcinogenicity in experimental animals.[8,9] However, recent results published by Ito and colleagues have shown that long-term dietary exposure to very high doses of BHA[10,11] induces forestomach papillomas and squamous cell carcinomas in F344 rats. Thus, it was clear that indiscriminate use of BHA may pose a safety problem.

The small aplocheilid fish *Rivulus ocellatus marmoratus* (synonym: *R. marmoratus*) is the only known vertebrate that naturally exhibits functional hermaphroditism with internal self-fertilization.[12] Consequently, a given population of this species is genetically homogeneous. Previous studies have shown that this fish is a useful model for *in vivo* carcinogenesis[13,14] and mutagenesis[15] due to this genetic character and the low cost of culture. In this connection, to improve our understanding of the carcinogenic potency of BHA, we evaluated the hepatocarcinogenicity of low dietary doses of BHA to this fish. We found a dose-dependent hepatic tumor induction by BHA in this species.

MATERIALS AND METHODS

Animal *Rivulus* was bred and reared in our aquariums as described previously.[13] Fish were housed in groups in separate 40-liter glass tanks. They were then kept in a room where the temperature was adjusted at 25±1°C and which was illuminated by fluorescent lamps for 14 h daily. Water of fish tanks was replaced with fresh water at 1-month intervals. Our fish stock has originated from a single individual obtained from the Zoologisches Institut and the Zoologisches Museum, University of Hamburg, in 1981.

BHA treatment BHA (Sigma, St. Louis, reagent grade, >99% purity) dissolved in absolute ethanol was mixed with freeze-dried chicken liver powder at concentrations of 0.01, 0.05, 0.2 and 0.8% (w/w), and then the ethanol was evaporated. The mixtures obtained in this way were used as the powdered diet. The liver powder was prepared from freshly slaughtered chickens in our laboratory with a freeze-drier (Labcon, Kansas City). To avoid possible contamination by carcinogenic mycotoxins in the diet, vegetable materials were not added, and the powdered diet was stored at −20°C.

Three-day-old fish larvae were fed the test diet containing various levels of BHA for 12 days *ad libitum*. The time course studies to determine the maximal biochemical effects of feeding BHA in rodents have shown that 12-day feeding of 0.75% BHA caused near maximal increases of the specific activities of hepatic microsomal enzymes responsible for xenobiotic metabolism.[16,17] Based on these experiences, we chose the duration of treatment period and dose levels of BHA. Control fish of the same age were given ethanol-treated chicken liver powder which did not contain BHA. Every day, diet which had not been completely consumed was removed and fresh diet was added to each fish tank daily. Active feeding of the test diet by larvae was observed, and the intake of BHA was confirmed by high-performance liquid chromatographic analysis of tissue homogenates of test fish.

Sampling and analysis To determine tumor incidences, fish were sampled at 6 months after the BHA treatments. Because the fish liver is small enough (about 4 mm in

[3] To whom reprint requests should be addressed.

diameter), the whole liver tissues were examined histologically. Every whole liver was fixed with Bouin's fluid, and serially sectioned. Liver slides were then stained with hematoxylin and eosin for histological diagnosis of tumor appearance. The histologic classification of liver tumors was based on criteria reported previously.[13]

RESULTS

Mortality The effect of different levels of BHA on the fish mortality is shown in Table I. Increasing the dietary BHA concentration had no effect on the mortality during the 6-month period following the BHA treatment.

Tumor incidence Hepatic tumor incidences induced by BHA are summarized in Table II. In all BHA-treated groups, there were significant increases of the tumor incidences. Surprisingly, about 10% of fish receiving a diet containing 0.01% BHA developed tumors, whereas none of the control fish did. This level of BHA in the diet is only half the level of 0.02% allowed in human foods by the United States Food and Drug Administration.[4] Results obtained with higher doses indicate a dose-dependent increase of tumor incidence.

Histology The control liver of this fish species consisted of a sheet-like arrangement of lipid-laden parenchymal cells with irregularly distributed vascular sinusoids (Fig. 1A). A uniformly sized round nucleus with a single large nucleolus was evenly distributed throughout all parenchymal cells.

The hepatic tumors were spherical or discoidal with an average diameter of 0.5–0.8 mm. Large tumors (about 3 mm) were also observed in some livers of fish treated with 0.8% BHA diet. The tumors were histologically similar to those induced by the hepatocarcinogen diethylnitrosamine (DENA) in *Rivulus*[13] and in medaka.[18] Most tumors were hepatocellular carcinomas with deeply basophilic cells (Fig. 1B). Generally large hyperchromatic nuclei and numerous mitotic figures were noted. Some consisted of broad cords of basophilic cells (Fig. 1B), whereas others showed basophilia with poorly differentiated trabecular structure (Fig. 1C, D).

Table I. Mortalities in *R. ocellatus marmoratus* during and after Dietary Administration of BHA

BHA dose (%)	No. of fish tested	No. of mortalities (%)	
		12 days[a]	6 months[b]
0	90	1 (1.1)	6 (6.7)
0.01	100	4 (4.0)	13 (13.5)
0.05	72	1 (1.4)	9 (12.7)
0.2	71	1 (1.4)	15 (21.4)
0.8	70	1 (1.4)	5 (7.3)

a) During 12 days of exposure.
b) During 6 months after exposure.

Table II. Incidence of Hepatic Tumors in *R. ocellatus marmoratus* after Dietary Exposure of BHA

BHA dose (%)	Tumor incidence[a] (%)
Control	0/83
0.01	8/83 (9.6)
0.05	11/62 (17.7)
0.2	12/55 (21.8)
0.8	31/64 (48.4)

a) No. of tumor-bearing fish/total No. of fish examined.

DISCUSSION

Earlier carcinogenicity tests showed that BHA induced tumors in both sexes of F344 rats[10, 11] and male Syrian golden hamsters.[19] The target organ was, however, limited to the forestomach, and the effective dose for induction of tumors was very high (1–2%). Exposure time was also very long (2 yr for rat, 1/2 yr for hamster). The BHA treatments did not induce any histopathological lesion in the liver of cynomogus monkeys (*Macaca fascicularis*)[20] or beagle dogs.[21] In view of the common use of BHA in the human diet, the carcinogenic potency of BHA should be evaluated at low doses in species without a forestomach to confirm the relevance of previous observations to humans. In spite of the low-dose and short-term treatment, hepatocarcinogenicity of BHA was demonstrated in the present study. The strength of BHA carcinogenicity at the highest dose (0.8%) is roughly comparable to that of 300 ppm DENA to *Rivulus*,[13] although the duration of DENA exposure was shorter (2 h) than that of BHA treatment. Thus, our results provide another line of evidence for the carcinogenicity of this food additive antioxidant to experimental animals.

R. ocellatus marmoratus has been demonstrated to be a very sensitive animal for hepatic tumor induction with hepatocarcinogens such as DENA[13] or aflatoxin B_1 (unpublished data). Again, with BHA, high sensitivity with a short latent period for liver tumor induction was also noticed. The reason for the high susceptibility of this fish to chemical carcinogens is unclear.

Since BHA does not show any mutagenic or clastogenic effect in various *in vitro* and *in vivo* assays,[4] the mechanism of the carcinogenic activity of this compound is unclear. The recently developed deletion assay[22] exhibits higher sensitivity to certain kinds of DNA-damaging agents which have been missed with other tests currently being used. Most nongenotoxic carcinogens were revealed to be in fact genotoxic in this assay system.[22]

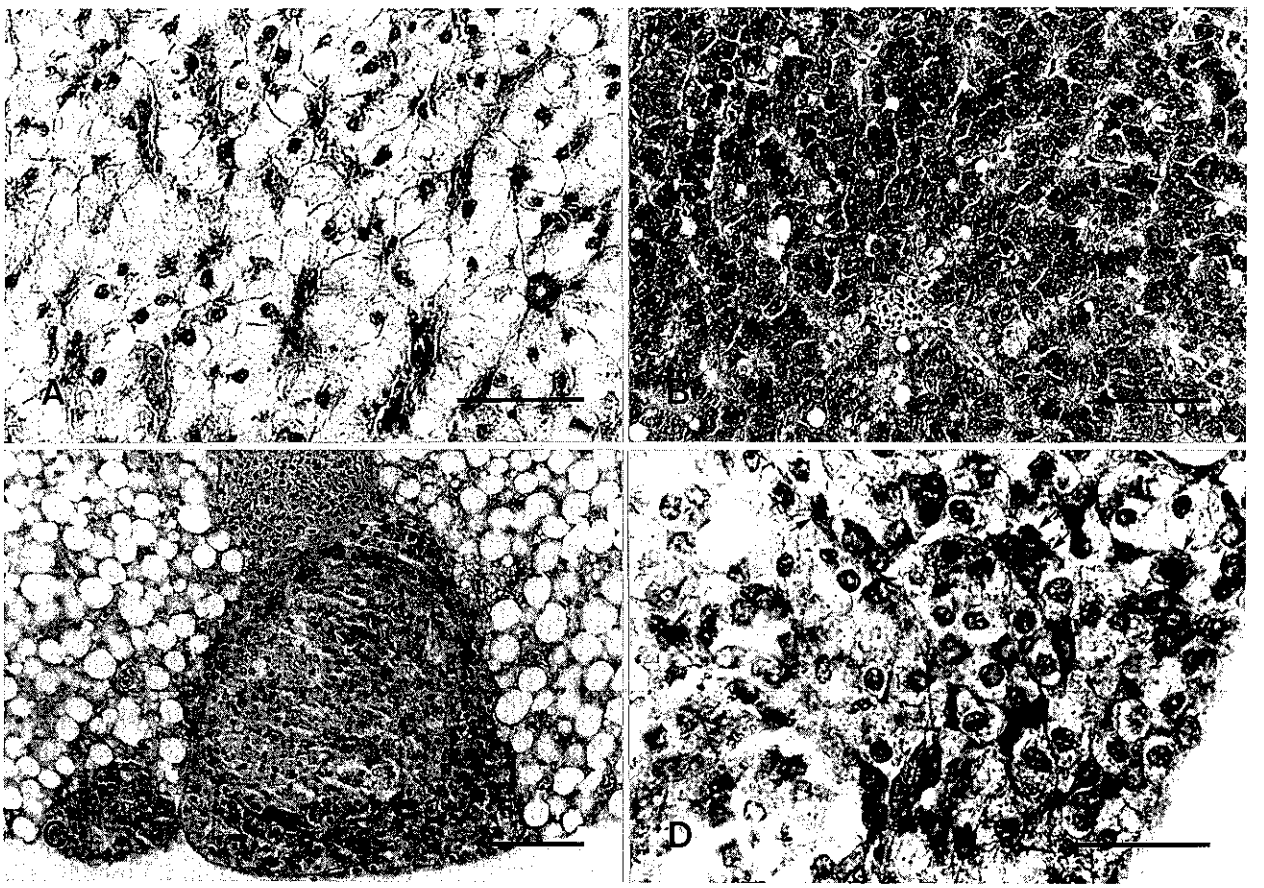

Fig. 1. Histology of control liver of adult *R. ocellatus marmoratus* (A). Liver tissue consisted of a sheet-like arrangement of parenchymal cells with irregularly distributed sinusoids. Section of a trabecular hepatocellular carcinoma from a fish fed a diet containing BHA (B). Note the deeply basophilic cells with increased nucleo-cytoplasmic ratio. Low power view of two tumor nodules in the highly lipid-laden liver from a fish fed a BHA-containing diet (C). High power view of Fig. 1C (D). Numerous mitotic figures (arrows) were visible. H-E stain. Bars; 50 μm (A, B), 60 μm (C), 20 μm (D).

Thus, genotoxicity of BHA should be re-examined with this test to improve our understanding of the mechanism of BHA carcinogenesis. DeStafney *et al.*[23] proposed that two factors may be of importance for BHA carcinogenesis. The first is thiol depletion resulting from direct binding of the quinone metabolites of BHA in the target tissue. The second is a direct attack on cellular constituents by reactive metabolites arising from the metabolism of BHA. The details of the responsible BHA metabolites and their target molecules, however, remain to be determined. Kroes and Wester[24] suggested that underlying hyperplastic activity of the forestomach may be crucial for the BHA-dependent forestomach carcinogenesis. Increased mitotic activity which occurs during hyperplasia could enhance the probability of an initiating agent, either exogenously derived or endogenously formed, attacking genetic material to an extent sufficient to induce a tumor. Thus, liver cell kinetic studies are required to show whether or not proliferative changes are induced by BHA in this fish. Since there is definite evidence for a modifying effect of BHA in two-stage carcinogenesis,[25] studies on promotion by BHA in this fish model would also be attractive.

ACKNOWLEDGMENTS

This work was supported by Korea Science and Engineering Foundation (KOSEF) grant 860411 to Prof. E.-H. Park.

(Received March 10, 1990/Accepted May 15, 1990)

REFERENCES

1) Castelli, M. G., Benfenati, E., Pastorelli, R., Salmona, M. and Fanelli, R. Kinetics of 3-*tert*-butyl-4-hydroxyanisole (BHA) in man. *Food Chem. Toxicol.*, **22**, 901–904 (1984).
2) Cummings, S. W., Ansari, G. A. S., Guengerich, F. P., Crouch, L. S. and Prough, R. A. Metabolism of 3-*tert*-butyl-4-hydroxyanisole by microsomal fractions in isolated rat hepatocytes. *Cancer Res.*, **45**, 5617–5625 (1985).
3) Fritsch, P., DeSaint-Blanquat, G. and Derache, R. Gastrointestinal absorption, in the rat, of anisole, *trans*-anethole, butylhydroxyanisole and safrole. *Food Cosmet. Toxicol.*, **130**, 359–364 (1975).
4) International Agency for Research on Cancer. "Monographs on the Evaluation of the Carcinogenic Risk of Chemicals on Humans. Vol. 30. Some Naturally Occurring and Synthetic Food Components, Furocoumarins and Ultraviolet Radiation," pp. 123–159 (1986). IARC, Lyon.
5) Batzinger, R. P., Ou, S. Y. and Bueding, E. Antimutagenic effects of 2(3)-*tert*-butyl-4-hydroxyanisole and of antimicrobial agents. *Cancer Res.*, **38**, 4478–4485 (1978).
6) Wattenburg, L. W. Inhibitors of chemical carcinogens. *Adv. Cancer Res.*, **26**, 197–226 (1978).
7) Williams, G. M., Tanaka, T. and Maeura, Y. Dose-related inhibition of aflatoxin B_1 induced hepatocarcinogenesis by the phenolic antioxidants, butylated hydroxyanisole and butylated hydroxytoluene. *Carcinogenesis*, **7**, 1043–1051 (1986).
8) Hodge, H. C., Fassett, D. W., Maynard, E. A., Downs, W. L. and Coye, R. D. Chronic feeding studies of butylated hydroxyanisole in dogs. *Toxicol. Appl. Pharmacol.*, **6**, 512–519 (1964).
9) Stoner, G. D., Shimkin, M. B., Kniazeff, A. J., Weisburger, J. H., Weisburger, E. K. and Gori, G. B. Test for carcinogenicity of food additives and chemotherapeutic agents by the pulmonary tumor response in strain A mice. *Cancer Res.*, **33**, 3069–3085 (1973).
10) 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).
11) Ito, N., Hagiwara, A., Shibata, M., Ogiso, T. and Fukushima, S. Induction of squamous cell carcinoma in the forestomach of F344 rats treated with butylated hydroxyanisole. *Gann*, **73**, 332–334 (1982).
12) Harrington, R. W., Jr. Oviparous hermaphroditic fish with internal self-fertilization. *Science*, **134**, 1749–1750 (1961).
13) Park, E.-H. and Kim, D. S. Hepatocarcinogenicity of diethylnitrosamine to the self-fertilizing hermaphroditic fish *Rivulus marmoratus* (Teleostomi: Cyprinodontidae). *J. Natl. Cancer Inst.*, **73**, 871–876 (1984).
14) Thiyagarajah, A. and Grizzle, J. M. Diethylnitrosamine-induced pancreatic neoplasms in the fish *Rivulus ocellatus marmoratus*. *J. Natl. Cancer Inst.*, **77**, 141–147 (1986).
15) Park, E.-H. and Yi, A.-K. Photoreactivation rescue and dark repair demonstrated in UV-irradiated embryos of the self-fertilizing fish *Rivulus ocellatus marmoratus* (Teleostei; Aplocheilidae). *Mutat. Res.*, **217**, 19–24 (1989).
16) Cha, Y.-N., Martz, F. and Bueding, E. Enhancement of liver microsome epoxide hydratase activity in rodents by treatment with 2(3)-*tert*-butyl-4-hydroxyanisole. *Cancer Res.*, **38**, 4496–4498 (1978).
17) Cha, Y.-N. and Heine, H. S. Comparative effects of dietary administration of 2(3)-*tert*-butyl-4-hydroxyanisole and 3,5-di-*tert*-butyl-4-hydroxytoluene on several hepatic enzyme activities in mice and rats. *Cancer Res.*, **42**, 2609–2612 (1982).
18) Ishikawa, T., Shimamine, T. and Takayama, S. Histologic and electron microscopy observations on diethylnitrosamine-induced hepatomas in small aquarium fish (*Oryzias latipes*). *J. Natl. Cancer Inst.*, **55**, 909–916 (1975).
19) Ito, N., Fukushima, S., Imaida, K., Sakata, T. and Masui, T. Induction of papilloma in the forestomach of hamsters by butylated hydroxyanisole. *Gann*, **74**, 459–461 (1983).
20) Iverson, F., Truelove, J., Nera, E., Lok, E., Clayson, D. B. and Wong, J. A 12-week study of BHA in the cynomolgus monkey. *Food Chem. Toxicol.*, **24**, 1197–1200 (1986).
21) Ikeda, G. J., Stewart, J. E., Sapienza, P. P., Peggins, J. O., Michel, T. C. and Olivito, V. Effect of subchronic dietary administration of butylated hydroxyanisole on canine stomach and hepatic tissue. *Food Chem. Toxicol.*, **24**, 1201–1221 (1986).
22) Schiestl, R. H. Nonmutagenic carcinogens induce intrachromosomal recombination in yeast. *Nature*, **337**, 285–288 (1989).
23) DeStafney, C. M., Prabhu, U. D. G., Sparnins, V. L. and Wattenburg, L. W. Studies related to the mechanism of 3-BHA-induced neoplasia of the rat forestomach. *Food Chem. Toxicol.*, **24**, 1149–1157 (1986).
24) Kroes, R. and Wester, P. W. Forestomach carcinogens; possible mechanism of action. *Food Chem. Toxicol.*, **24**, 1083–1089 (1986).
25) Ito, N., Fukushima, S. and Tsuda, H. Carcinogenicity and modification of the carcinogenic responses by BHA, BHT, and other antioxidants. *Crit. Rev. Toxicol.*, **15**, 109–150 (1985).