

Original

Ethanol Does Not Promote MeIQx-initiated Rat Colon Carcinogenesis Based on Evidence from Analysis of a Colon Cancer Surrogate Marker

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Abstract: Epidemiological studies suggest that alcohol consumption increases the risk of developing colorectal cancer. However, the data are confounded by numerous cosegregating variables. To cast further light on the relationships between alcohol intake and colon cancer development, 21-day-old male F344/DuCrj rats were fed 200 ppm 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) in their diet for 8 weeks and doses of 0, 0.1, 0.3, 1, 3, 10 and 20% of ethanol in their drinking water *ad libitum* for 16 weeks thereafter. The rats were sacrificed after 24 weeks of experiment, and aberrant crypt foci (ACF), surrogate lesions for colon cancer, were examined under a light microscope at low magnification. Ethanol was found not to affect the ACF formation at any dose compared with the initiated-controls. Furthermore, ethanol did not alter colon epithelial cell proliferation. These data, obtained by analysis of a colon cancer surrogate marker lesion, indicate that ethanol lacks promotion activity for MeIQx-initiated rat colon carcinogenesis. (J Toxicol Pathol 2009; 22: 65–70)

Key words: ethanol, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), rat colon carcinogenesis

Introduction

There is abundant epidemiological evidence showing that excessive and chronic alcohol consumption contribute to cancer development in organs such as the oral cavity, pharynx, larynx, esophagus, liver and breast¹. The epidemiologic data concerning the association between alcohol consumption and colorectal cancer are not as clear as those concerning cancers of the upper aerodigestive tract. Most studies, however, have detected a positive correlation². In 1999, a consensus conference of the World Health Organization on Nutrition and Colorectal Cancer concluded that chronic alcohol ingestion, even with low daily intake (one to three drinks or 10 to 40 g per day), results in a 1.5- to 3.5-fold increase in risk of rectal cancer and a lesser increase in risk of colonic cancer in both sexes³. This conclusion also gained support at a meeting on alcohol and cancer at the

International Agency for Research on Cancer⁴.

Experimental animal studies are critical in clarifying the role of alcohol in organ carcinogenesis. It has been reported that ethanol per se does not have carcinogenic activity even after lifelong exposure in rodents⁵. The hypothesis is therefore that alcohol may act as a co-carcinogen or tumor-promoter for colon carcinogenesis¹. However, experimental data on alcohol and colorectal cancer are controversial and complex, depending on the experimental design^{6–8}. In general, it can be concluded that alcohol stimulates colorectal carcinogenesis primarily during the preinitiation and initiation phases, as interaction may occur between ethanol and procarcinogenic metabolism in these phases².

Heterocyclic amines (HCAs) are ubiquitously present in cooked foods and are known to form DNA adducts in experimental animals and human⁹. Recent epidemiological studies have indicated that increased risk of colorectal cancer development is associated with high intake of red meat and HCAs, such as 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx)^{10,11}. MeIQx is strongly mutagenic in the Ames test¹² and is carcinogenic in rodents when fed at high doses, inducing tumors in the livers, Zymbal glands,

Received: 26 August 2008, Accepted: 3 December 2008

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skin and clitoral glands of rats at a dose of 400 ppm in their diet¹³ and in the livers, lungs, and hematopoietic systems of mice at a dose of 600 ppm¹⁴. It has also been documented that MeIQx induces aberrant crypt foci (ACFs), surrogate marker lesions for colon cancers, in rats¹⁵ and mice¹⁶.

Exposure of humans to HCAs is all but inescapable, and many people drink alcohol; however, only a limited amount of data are available concerning the effects of interaction between HCAs and alcohol with regard to colon carcinogenesis, especially from *in vivo* experiments. In the present study, we therefore examined the potential for the ability of ethanol to promote MeIQx-induced colon carcinogenesis in rats. For this purpose, we focused on ACFs in tissue samples from a previous study that demonstrated dose-dependent promotion of MeIQx-induced hepatocarcinogenesis by ethanol in male rats¹⁷.

Materials and Methods

Chemicals and antibodies

Ethanol (99.5%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and MeIQx was purchased from the Nard Institute (Osaka, Japan). Mouse anti-proliferating cell nuclear antigen (PCNA) monoclonal antibodies were obtained from Dako (Carpinteria, CA, USA).

Animals

A total of 125 male, 20-day-old, F344/DuCrj rats were purchased from Charles River Japan (Atsugi, Japan). Their housing conditions and treatment were as described previously¹⁷.

Ethics

The experiments were conducted in accordance with the guidelines for Animal Experiments at Osaka City University Medical School, which are in line with other domestic and international guidelines and laws concerning animal rights.

Experimental design

One hundred and twenty-five, 21-day-old, F344/DuCrj rats were divided into eleven experimental groups. The numbers of rats in the groups were 15 (groups 1 to 7) or 5 (groups 8 to 11) per group. In groups 1 to 7 and 11, the rats were fed MF pellet diet mixed with 200 ppm MeIQx (Oriental Yeast Co., Ltd., Tokyo, Japan), and water was provided *ad libitum* for the first 8 weeks; groups 8 to 10 were fed MF pellet diet without MeIQx. The animals in groups 10 and 11 were euthanized at experimental week 8. Thereafter, the remaining rats received doses of 0 (groups 1 and 8), 0.1 (group 2), 0.3 (group 3), 1 (group 4), 3 (group 5), 10 (group 6) and 20% ethanol (vol/vol; groups 7 and 9) in their drinking water and MF pellet diet *ad libitum* for 16 weeks. During the period of ethanol administration, the drinking water was changed six times per week. At experimental week 24, all rats were euthanized after overnight withdrawal

of food. At sacrifice, the animals were anesthetized with diethyl ether, and their colons were quickly removed. After evaluation of ACF, two samples of the proximal, middle and distal colon were cut into strips and routinely processed for embedding in paraffin. Sections were stained with hematoxylin and eosin (H&E) for histopathological and immunohistochemical examination. Diagnosis and classification of tumors and hyperplasias were performed according to the nomenclature for classification of colon tumors and preneoplastic lesions in the rat proposed by IARC¹⁸. The hyperplastic lesions recognized in the present study were all atypical hyperplasias and did not include a focal or reactive hyperplasia.

ACF counts

Colons were quickly excised, flushed with saline, inflated by intraluminal injection of 10% phosphate-buffered formalin solution, slit open along the longitudinal median axis from the cecum to anus and fixed flat between two pieces of filter paper in 10% phosphate-buffered formalin. After fixation for at least 24 h at 4°C, all colons were stained with 0.2% methylene blue (in H₂O) for 3–5 min and then examined for ACF by light microscopy at 40× and 100× magnification using the following criteria for identification: (1) increased size compared with normal crypts, (2) enlarged pericryptal zone, (3) slight elevation above the surrounding mucosa and (4) more frequent occurrence of an elongated luminal opening. ACFs were assessed for the number of aberrant crypts in each focus (1 crypt, 2 crypts, 3 crypts and ≥4 crypts).

Immunohistochemistry for PCNA

Immunoenzymatic staining for PCNA was performed after sequential treatment with 3% H₂O₂, exposure to an anti-PCNA mouse monoclonal antibody at room temperature for 1 hr and then horseradish peroxidase coupled to an inert polymer backbone (Dako EPOS, Dako Cytomation, Carpinteria, CA, USA). The sites of peroxidase binding were demonstrated by DAB. More than 500 epithelial cells of well-visualized crypts from normal-appearing mucosa were counted per colonic segment under a light microscope. PCNA indices were estimated as the numbers of positive nuclei per 100 epithelial cells (%). In groups 1 to 7, 6 animals were evaluated. Immunohistochemical evaluation of PCNA was performed in a blind manner.

Statistical analysis

Statistical analysis was performed with Statistical Analysis System (SAS) version 9.1.3 (SAS Institute Incorporated, Cary, North Carolina, USA). For the incidences of colon tumors, hyperplasias and ACFs, the Fisher's exact probability test was employed. For experiments using single dose levels of MeIQx or ethanol, the data for each group were first analyzed with the F-test for homogeneity of variance. If homogeneous, the data were analyzed by the Student's t test, and if not, they were by the Aspin-Welch's t test. For the experiment using multiple

Table 1. Body Weight, Food Consumption, Water Intake and Ethanol Intake Data for Rats Treated with MeIQx (8 Weeks) and/or Ethanol (16 Weeks)

Group	Treatment	No. of rats (terminal)	Final body weight (g)	Average food consumption (g/head/day)	Average water intake (g/head/day)	Average ethanol intake (g/kg B.W./day)
1	MeIQx alone	15	385 ± 18	13.0 ± 0.3	20.0 ± 1.4	0
2	MeIQx →0.1% EtOH	15	383 ± 20	13.1 ± 0.7	20.7 ± 0.9	0.06 ± 0.00
3	MeIQx →0.3% EtOH	15	378 ± 21	13.2 ± 0.6	20.4 ± 0.8	0.18 ± 0.01
4	MeIQx →1% EtOH	15	372 ± 25	12.9 ± 0.3	21.0 ± 0.8*	0.64 ± 0.05
5	MeIQx →3% EtOH	15	379 ± 21	12.7 ± 0.5*	20.6 ± 0.9	1.83 ± 0.09
6	MeIQx →10% EtOH	15	364 ± 19*	11.0 ± 0.3**	18.1 ± 0.5**	5.37 ± 0.23
7	MeIQx →20% EtOH	15	322 ± 15**	8.8 ± 0.2**	15.0 ± 0.2**	9.10 ± 0.50
8	Control	5	375 ± 13	13.2 ± 0.8	19.4 ± 0.4	0
9	20% EtOH	4	309 ± 16 ^{##}	9.1 ± 1.5 ^{##}	14.0 ± 0.3 ^{##}	8.60 ± 0.50
10 [†]	No treatment	5	276 ± 7	11.7 ± 0.0	19.3 ± 0.1	–
11 [†]	MeIQx alone	5	254 ± 12 [§]	11.4 ± 0.1	20.1 ± 1.3	–

Values are represented as means ± SD.

[†] The rats in groups 10 and 11 were sacrificed in experimental week 8 before ethanol administration.

* Significantly different from group 1 ($P < 0.05$).

** Significantly different from group 1 ($P < 0.01$).

^{##} Significantly different from group 8 ($P < 0.01$).

[§] Significantly different from group 10 ($P < 0.05$).

dose levels of ethanol, the data were initially tested for homogeneity using Bartlett's analysis. For data found to be homogeneous, Dunnett's multiple analysis was performed. If the data were not homogeneous, Steel's multiple analysis was conducted. The significance of differences was determined based on probability levels of 1% and 5%. The data are presented as means or means ± standard deviation (SD).

Results

Final body weights, food consumption and water intake

At week 8, all rats were in good general condition, and there were no significant differences among the groups with regard to food consumption and water intake (data not shown). After week 8, food consumption of the rats in Groups 5, 6 and 7, which were administered a dose of 3, 10 or 20% ethanol, was significantly decreased compared with the initiated control Group 1 (Table 1). Furthermore, water intake of the rats in Groups 6 and 7, which were administered a doses of 10 and 20% ethanol, was significantly decreased compared with the initiated control Group 1. In Group 4, water intake was significantly increased compared with the initiated control Group 1, but there was no dose-dependence. Therefore, the increased water intake in Group 4 was not considered to have any toxicological implications. One non-initiated rat administered 20% ethanol (Group 9) died in experimental week 11, and emaciation was observed at autopsy. The final body weights of rats in Groups 6 and 7 were significantly reduced compared with Group 1. In the groups without MeIQx-treatment (Group 8 and 9), administration of 20% ethanol also reduced final body weight, food consumption and water intake (Table 1).

Effects of ethanol on ACF formation

At week 8, ACFs were observed in all MeIQx-treated rats (Group 11); however, no ACFs were observed in the no-treatment rats (Group 10; Table 2). At experimental week 24, the animals treated with MeIQx developed ACFs without relation to ethanol treatment (Fig. 1A–D). The incidences and multiplicities of ACFs in rats administered doses of 0.1 to 10% ethanol (Groups 2 to 6) were not different from those of the control Group 1 (Table 2). In the 20% ethanol treatment group (Group 7), there were no differences in the incidences of any type of ACF compared to the control group (Group 1). There was no difference in ACF multiplicity between Group 7 and the control group with exception of a significant decrease in ACFs with 3 crypts. No statistically significant differences in formation of ACFs were observed between the non-treatment (Group 8) and ethanol only treatment groups (Group 9).

Effects of ethanol on formation of proliferative lesions

In animals administered doses of 0.1, 0.3, 10 and 20% ethanol after MeIQx treatment, a few hyperplasias and adenomas were observed in the colon at experimental week 24. However, there was no relationship between formation of hyperplastic and neoplastic lesions and ethanol intake (Table 3).

Evaluation of cell proliferation by immunohistochemistry for PCNA

The results of immunohistochemical staining for PCNA, a marker for cell proliferation, are presented in Table 3. The PCNA-positive indices for the colon mucosa did not differ between the ethanol treatment groups at any dose and the initiated controls. Ethanol also had no effect on cell proliferation in the non-MeIQx treatment groups.

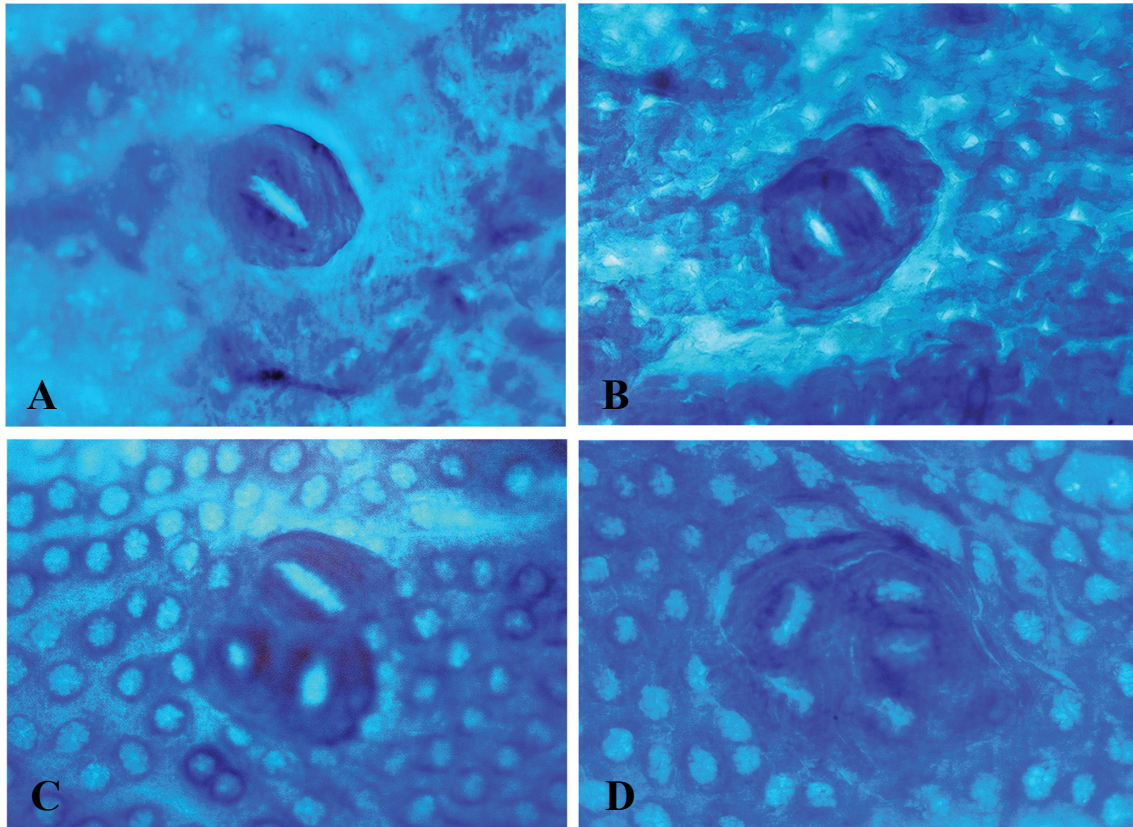


Fig. 1. Formation of aberrant crypt foci (ACFs) in the colons of F344 rats administered ethanol for 16 weeks after MeIQx initiation for 8 weeks. A-D show typical ACFs consisting of 1 Crypt (A), 2 Crypts (B), 3 Crypts (C) and 4 Crypts or more (D). The samples are stained with 0.2% methylene blue stain. The original magnification is 100 \times .

Table 2. Formation of Aberrant Crypt Foci (ACF) in Rats Treated with MeIQx (8 Weeks) and/or Ethanol (16 Weeks)

Group	Treatment	n	1 crypt		2 crypts		3 crypts		≥ 4 crypts		Total	
			Incidence (%)	Multiplicity (No./animal)	Incidence (%)	Multiplicity (No./animal)	Incidence (%)	Multiplicity (No./animal)	Incidence (%)	Multiplicity (No./animal)	Incidence (%)	Multiplicity (No./animal)
1	MeIQx alone	15	80	1.7 \pm 1.3	87	2.5 \pm 1.5	93	2.3 \pm 1.3	87	3.5 \pm 2.9	100	9.9 \pm 4.7
2	MeIQx →0.1% EtOH	15	50	1.3 \pm 1.6	93	2.3 \pm 1.2	79	1.5 \pm 1.3	100	4.4 \pm 2.1	100	9.5 \pm 4.1
3	MeIQx →0.3% EtOH	15	42	1.0 \pm 1.4	79	1.7 \pm 1.4	86	1.7 \pm 1.2	86	2.1 \pm 1.4	100	6.5 \pm 2.8
4	MeIQx →1% EtOH	15	57	1.1 \pm 1.3	79	2.5 \pm 2.0	100	2.0 \pm 1.2	93	3.9 \pm 3.1	100	9.5 \pm 3.8
5	MeIQx →3% EtOH	15	50	0.9 \pm 1.4	86	2.1 \pm 1.3	93	2.1 \pm 1.1	100	3.9 \pm 2.1	100	9.0 \pm 3.1
6	MeIQx →10% EtOH	15	71	1.2 \pm 1.4	71	1.6 \pm 1.5	86	1.3 \pm 1.0	100	3.1 \pm 1.5	100	7.2 \pm 3.0
7	MeIQx →20% EtOH	15	69	1.2 \pm 1.2	77	2.2 \pm 1.6	54	0.9 \pm 1.2*	69	2.5 \pm 2.3	100	6.8 \pm 2.6
8	Control	5	20	0.2 \pm 0.5	40	0.4 \pm 0.8	40	0.6 \pm 1.3	40	0.4 \pm 0.8	80	1.6 \pm 2.8
9	20% EtOH	4	0	0	0	0	0	0	50	0.5 \pm 0.8	50	0.5 \pm 0.8
10 [†]	No Treatment	5	0	0	0	0	0	0	0	0	0	0
11 [†]	MeIQx 200 ppm	5	20	0.2 \pm 0.5	80	1.2 \pm 0.8 [#]	60	0.6 \pm 0.6	60	0.6 \pm 0.6	100 [#]	2.6 \pm 0.9 [#]

Multiplicity values are presented as means \pm SD.

[†] The rats in groups 10 and 11 were sacrificed in experimental week 8 before ethanol administration.

* Significantly different from group 1 ($P < 0.05$).

[#] Significantly different from group 10 ($P < 0.05$).

Table 3. Formation of Hyperplasia and Adenoma and Cell Proliferation Indices in Rats Treated with MeIQx (8 Weeks) and/or Ethanol (16 Weeks)

Group	Treatment	n	Hyperplasia		Adenoma		PCNA positive cell index (%)
			Incidence (%)	Multiplicity (No./animal)	Incidence (%)	Multiplicity (No./animal)	
1	MeIQx alone	15	0	0	0	0	10.3 ± 1.1
2	MeIQx →0.1% EtOH	15	6.7	0.07 ± 0.13	0	0	10.1 ± 1.7
3	MeIQx →0.3% EtOH	15	6.7	0.07 ± 0.13	6.7	0.07 ± 0.13	9.8 ± 1.2
4	MeIQx →1% EtOH	15	0	0	0	0	9.7 ± 0.7
5	MeIQx →3% EtOH	15	0	0	0	0	11.1 ± 1.2
6	MeIQx →10% EtOH	15	0	0	13.3	0.25 ± 0.35	9.7 ± 1.1
7	MeIQx →20% EtOH	15	13.3	0.25 ± 0.35	6.7	0.07 ± 0.13	10.4 ± 0.9
8	Control	5	0	0	0	0	9.5 ± 1.2
9	20% EtOH	4	0	0	0	0	9.8 ± 0.7

Multiplicity values are presented as means ± SD.

Discussion

In past epidemiological studies, it has been proposed that almost 90% of large intestinal cancers might be caused by dietary factors¹⁹. Many researchers have pointed to relationships between dietary habits and colon cancer. HCAs and ethanol itself could be direct risk factors for human colon cancer, with other factors (fruits, vegetables, fiber and fecal weight) implicated as modulating their influences^{2,20,21}. However, in the present study, we could not find any evidence that ethanol promotes MeIQx-induced ACF formation, although it has previously been found to promote the formation of hepatocellular adenomas and/or carcinomas at doses of 3% or more via induction of cytochrome P450 2E1 (CYP 2E1)¹⁷. Previous studies of colorectal cancer using carcinogen-treated (azoxymethane or 1,2-dimethylhydrazine) rodent models have also suggested that ethanol does not promote colon carcinogenesis^{7,8}.

On the other hand, ethanol may be considered a co-carcinogen, and the mechanisms of colorectal carcinogenesis stimulation of ethanol could well be related to metabolism of procarcinogens². To clarify the effects of interaction between HCAs and ethanol in colon carcinogenesis, further studies after treatment with potent colon carcinogenic HCAs, PhiP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) or MeIQ (2-amino-3-methylimidazo[4,5-*f*]quinoline), are needed.

In the present study, ACF formation seemed to be affected in the 20% ethanol treatment group (Group 7) compared with the MeIQx-alone group (Group 1). The multiplicity of 3 crypt ACFs in Group 7 was significantly decreased, although other findings, such as the incidences of ACF, tumor formation and cell proliferation, were not affected. A similar tendency without statistical significance

was observed for formation of ACFs in the ethanol only treatment group (Group 9) compared with the non-treatment group (Group 8). Several studies have revealed that food restriction inhibits development of ACFs and colon tumors^{22,23}. In this context, it is interesting that food consumption and final body weight were significantly decreased in the 20% ethanol treatment groups (Group 7 and 9). These data suggest that the cause of decreased ACF formation was body weight loss due to decreased food intake, rather than the ethanol itself. Indeed, it remains a possibility that body weight loss nullified any promotion effects of ethanol on ACF formation. To clarify whether calorie restriction affects the effects of ethanol on ACF formation, further studies with an isocaloric diet are needed.

In conclusion, the present analysis of a surrogate marker for colon cancer indicates that ethanol has no promotional effect on MeIQx-initiated rat colon carcinogenesis, although it does promote rat hepatocarcinogenesis¹⁷.

Acknowledgments: This investigation was supported by a grant from the Ministry of Health, Labour and Welfare of Japan. We acknowledge the expert technical support of Kaori Touma and other technicians in the histological examination.

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