# Inhibitory Effect of Ellagic Acid on N-2-Fluorenylacetamide-induced Liver Carcinogenesis in Male ACI/N Rats

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The effect of ellagic acid (EA) on the hepatocarcinogenesis induced by N-2-fluorenylacetamide (FAA) was investigated in male ACI/N rats. Rats were fed diet containing 200 ppm FAA and 400 ppm EA for 16 weeks, and diet containing 400 ppm EA alone was fed to the animals for one week before FAA exposure and one week after the carcinogen treatment. Animals were killed at intervals up to 20 weeks after cessation of the carcinogen. Liver altered foci and neoplasms were quantified using γ-glutamyl transpeptidase reaction as well as conventional staining for identification. Exposure to FAA alone induced a substantial number of altered foci and at the end of experiment (week 36), the incidence of hepatocellular neoplasms was 100%. In the group receiving EA together with FAA, the number of altered foci was decreased at all time points and at termination, the final incidence of hepatocellular neoplasms (30%) was also reduced. Thus, EA inhibited the hepatocarcinogenesis induced by FAA when it was administered concurrently with the carcinogen.

Key words: Inhibitory effect — Ellagic acid — Hepatocarcinogenesis — N-2-Fluorenyl-acetamide — Rats

It is well known that dietary factors influence the process of carcinogenesis. Some natural products have been described as chemopreventive agents that inhibit the effect of several chemical carcinogens.[1] Ellagic acid (EA), a naturally occurring phenol,[2] has been reported to inhibit benzo[a]pyrene 7,8-dihydrodiol-9,10-epoxide-induced mutagenicity in *Salmonella typhimurium* TA100 and Chinese hamster V79 cells.[3] Also, EA inhibits aflatoxin $B_1$ mutagenesis in *Salmonella typhimurium* TA100 and DNA damage in cultured rat and human tracheobronchial tissues.[4] The development of skin tumors induced by 3-methylcholanthrene was inhibited by administration (painting or in drinking water) of EA to mice[5, 6] and EA inhibited benz[a]pyrene-induced lung adenomas in mice when administered either in the diet or ip.[7] Thus, EA has been shown to inhibit the mutagenicity and carcinogenicity of ultimate carcinogens or chemicals that require metabolic activation in order to exert their carcinogenicity. Recently, it was reported that EA enhances glutathione S-transferase activity in the mouse liver.[8] Teel[9] found that kidney and

liver contain the highest amounts of radioactivity in mice given [$^3$H]EA. Therefore, it is suspected that EA has a modifying effect on liver carcinogenesis. Since EA is present in grapes, strawberries, raspberries and certain nuts[2, 10–12] which are normally consumed by humans,[11] it is of interest to evaluate the effect of EA on carcinogenesis in rodents.

In the present study, the potential inhibitory effect of EA on N-2-fluorenylacetamide (FAA)-induced liver carcinogenesis was examined in male ACI/N rats.

## MATERIALS AND METHODS

**Animals**  Male inbred ACI/N rats, which have been maintained in our laboratory, were used. At 6 weeks of age, these rats were transferred to the holding room and randomized into experimental and control groups. Rats were housed 3 or 4 to a wire cage. The holding room was maintained at $23 \pm 2°$, $50 \pm 10\%$ humidity, and a 12 hr light-12 hr dark cycle.

**Chemicals**  EA ($>97\%$ pure) was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo. FAA was obtained from Nakarai Chemicals, Ltd., Kyoto.

**Treatment of Animals** (Fig. 1)  A total of 70 rats were divided into four groups as shown in the tables. Nineteen rats in group 1 were fed the diet
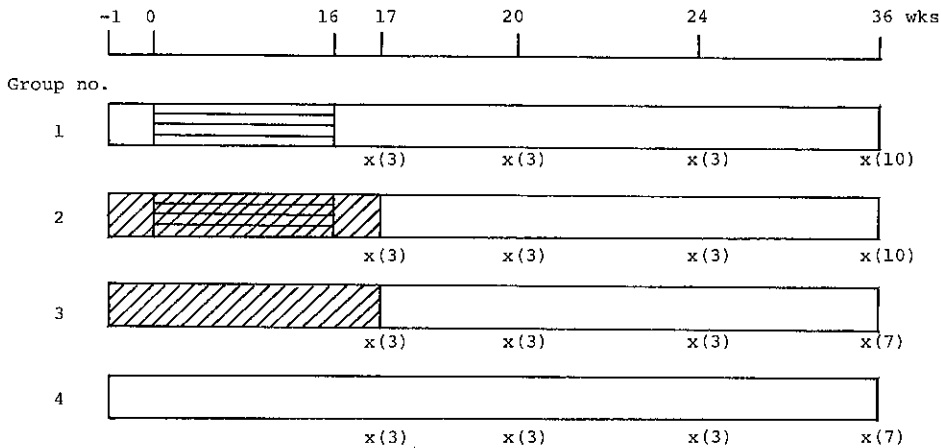
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Fig. 1.  Experimental protocol. ▤, 200 ppm FAA; ▨, 200 ppm FAA+400 ppm EA; ▨, 400 ppm EA; ☐, basal diet CE-2; x, sacrifice (no. of rats).

containing 200 ppm FAA for 16 weeks and maintained on the basal diet, CE-2 (CLEA Japan, Tokyo) for 20 weeks. Nineteen rats in group 2 were fed the diet containing 200 ppm FAA and 400 ppm EA for 16 weeks. These rats were fed 400 ppm EA for one week before and after the carcinogen administration, respectively and then maintained on the basal diet for 19 weeks. Sixteen rats in group 3 were fed diet containing 400 ppm EA beginning one week before the start of the study and continuing for 18 weeks, and then maintained on the basal diet for 19 weeks. Sixteen rats in group 4 were fed only the basal diet during the entire experimental period of 36 weeks and served as controls. Diets were prepared every 4 weeks and stored at 4° in a cold room. Determination of the concentration of FAA or EA was not done during the experiment because these chemicals are quite stable.

The animals were carefully observed and weighed weekly. At weeks 17, 20 and 24, three animals from each group were killed to determine the inhibitory effect of EA on the development of altered liver cell foci. At the end of the experiment (week 36), the remaining animals were killed to determine the final incidence of liver neoplasms. Complete autopsies were performed on all animals. At autopsy, the livers were removed and weighed, and slices were taken from each sublobe.[13] One slice was fixed in 10% buffered formalin and another was fixed in 95% cold ethanol (4°) and embedded in soft paraffin at a temperature below 55° for $\gamma$-glutamyl transpeptidase (GGT) reaction.[14] Two serial 5 $\mu$m sections were cut, and one section was stained with hematoxylin and eosin (H-E) and the other incubated for GGT reaction

at room temperature with a substrate.[14] Hepatocellular altered foci, liver cell adenomas and carcinomas were diagnosed according to the criteria of the Institute for Laboratory Animal Resources monograph on histologic typing of liver tumors.[15] The incidence of altered liver cell foci was quantified on the H-E-stained and GGT-reacted sections using a microscope and expressed as number of foci/cm².

Differences between the groups at various time points were tested for significance using Student's $t$ test. The incidence and multiplicity of liver cell tumors (adenomas and carcinomas) in each group were compared using Fisher's exact probability test and Student's $t$ test, respectively.

## RESULTS

The average body weight gains and relative liver weights in each group at the end of the experiment are shown in Table I. The body weights of rats in all groups were similar. Administration of EA to rats in group 3 did not affect the body weights or liver weights compared to the controls in group 4. Treatment with FAA increased the liver weights in group 1 due to the occurrence of tumors. The liver weights of rats in group 2 (FAA+EA) were significantly higher than those in group 3 (EA alone), but were slightly lower than those of rats in group 1 (FAA alone) without statistical significance.

The dietary administration of FAA induced a substantial number of liver cell altered foci

Table I.  Effect of EA and FAA on Body and Liver Weights of Male Rats at the End of the Experiment

| Group no. | Treatment | No. of rats | Body wt. (g) | Liver wt. (g) | Relative liver wt. (g/100 g body wt.) |
|---|---|---|---|---|---|
| 1 | FAA alone | 10 | $246 \pm 24^{a)}$ | $11.7 \pm 1.3^{b)}$ | $4.75 \pm 0.31^{c)}$ |
| 2 | FAA + EA | 10 | $269 \pm 18$ | $10.9 \pm 0.4^{d)}$ | $4.06 \pm 0.34^{d)}$ |
| 3 | EA alone | 7 | $277 \pm 8$ | $8.7 \pm 1.3$ | $3.15 \pm 0.43$ |
| 4 | Basal diet | 7 | $263 \pm 24$ | $8.8 \pm 1.7$ | $3.38 \pm 0.61$ |

a) Mean ± SD.
b, c) Significantly different from group 4 by Student's $t$ test ($b$, $P < 0.05$; $c$, $P < 0.001$).
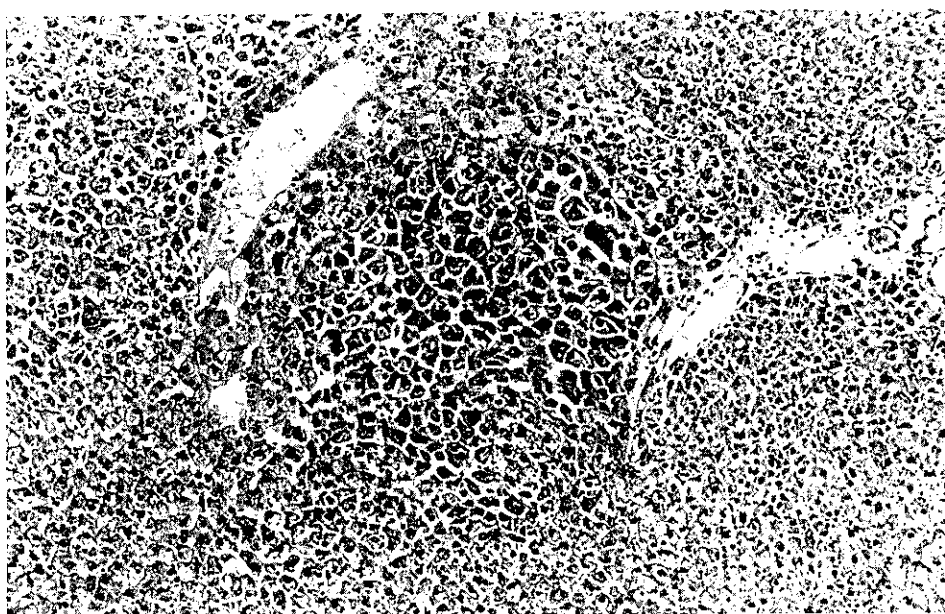d) Significantly different from group 3 by Student's $t$ test ($P < 0.001$).



Fig. 2.  An eosinophilic hepatocellular altered focus in a rat of group 1. H-E stain, ×510.

(Fig. 2) detectable in terms of GGT activity and liver cell neoplasms (Figs. 3 and 4) as shown in Table II. After 16 weeks of FAA exposure, an average of 39.49 foci on the H-E-stained sections and 45.67 GGT-positive foci on the GGT-reacted specimens were present in group 1. By 8 weeks after the cessation of FAA exposure, the incidence of foci in group 1 was increased but did not increase further by the end of the experiment, probably due to the extensive replacement of liver by neoplasms. The number of foci in group 2 was almost half of that in group 1 during and at the end of the study. There were no liver cell foci in rats of groups 3 and 4 throughout the experiment.

The incidence of hepatocellular neoplasms (adenomas and carcinomas) is shown in Table III. There were no liver cell neoplasms in groups 3 and 4. Exposure to FAA alone (group 1) induced liver cell neoplasms in 100% of rats, with a multiplicity of 4.0 neoplasms/rat. Feeding of EA together with FAA (group 2) reduced the liver cell neoplasm incidence to only 30% and the multiplicity to 0.6/rat at the end of the study. The
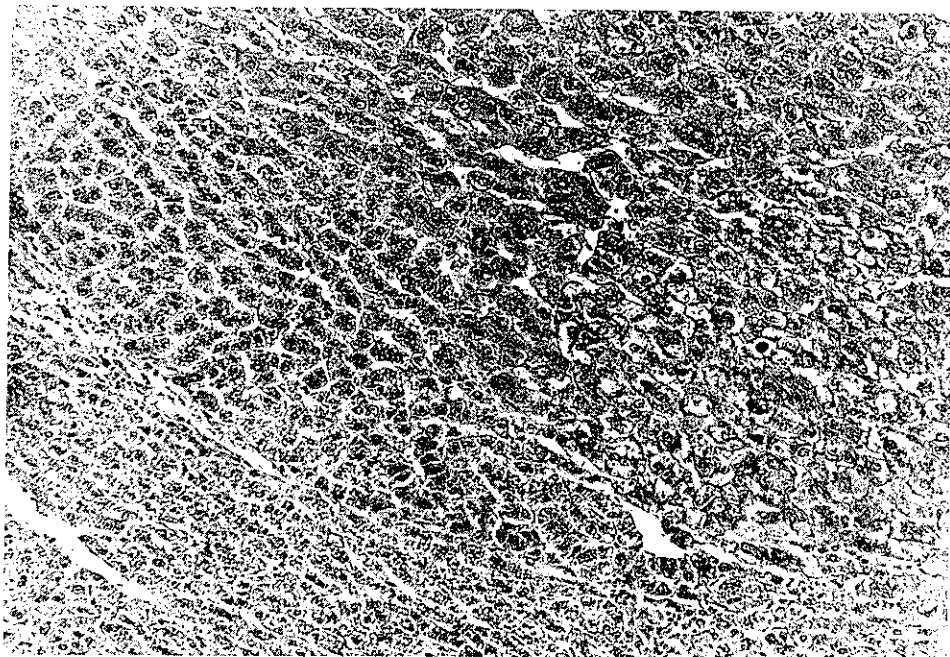
Fig. 3. A hepatocellular adenoma in a rat of group 1. H-E stain, ×510.
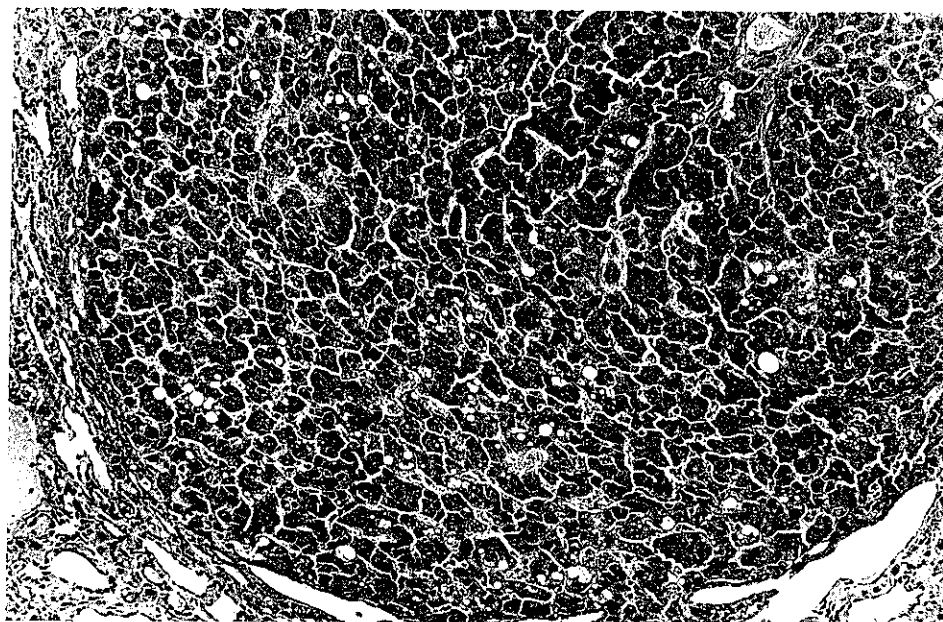


Fig. 4. A hepatocellular carcinoma in a rat of group 1. H-E stain, ×510.

Table II. Effect of EA on the Incidence of Altered Liver Cell Foci Induced by FAA

| Group no. | Treatment | Incidence of altered liver cell foci (no./cm² of liver) at: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 16 wk | | 20 wk | | 24 wk | | 36 wk | |
| | | H-E | GGT (+) | H-E | GGT (+) | H-E | GGT (+) | H-E | GGT (+) |
| 1 | FAA alone | 39.49[a] ±7.95 | 45.67 ±5.44 | 52.32 ±4.29 | 54.00 ±0.82 | 54.83 ±2.62 | 55.67 ±3.30 | 52.02 ±7.93 | 59.80 ±6.34 |
| 2 | FAA+EA | 16.45[b] ±5.87 | 21.00[b] ±5.35 | 18.21[c] ±2.23 | 20.00[c] ±4.08 | 22.42[c] ±3.37 | 23.67[c] ±4.19 | 24.49[c] ±3.46 | 24.33[c] ±0.94 |
| 3 | EA alone | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | Basal diet | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

a) Mean±SD.
b, c) Significantly different from group 1 by Student's t test (b, $P<0.05$; c, $P<0.001$).

Table III. Effect of EA on the Occurrence of Hepatic Neoplasms Induced by FAA

| Group no. | Treatment | Incidence (%) | No. of rats with adenomas (no. of adenomas) | No. of rats with carcinomas (no. of carcinomas) | Multiplicity |
|---|---|---|---|---|---|
| 1 | FAA alone | 10/10 (100) | 7(20) | 8(20) | 4.00±2.41 |
| 2 | FAA+EA | 3/10[a] (30) | 2[b](3) | 2[c](3) | 0.60±1.02[d] |
| 3 | EA alone | 0/7 (0) | 0 | 0 | 0 |
| 4 | Basal diet | 0/7 (0) | 0 | 0 | 0 |

a–c) Significantly different from group 1 by Fisher's exact probability test (a, $P<0.002$; b, $P<0.04$; c, $P<0.01$).
d) Significantly different from group 1 by Student's t test ($P<0.001$).

numbers of rats with liver cell tumors (number of tumors) in group 1 were 1/3 rats (1 adenoma) at week 16, 1/3 rats (3 adenomas) at week 20 and 3/3 rats (4 adenomas and 3 carcinomas) at week 24 and those in group 2, 1/3 rats (1 adenoma) at week 16, 1/3 rats (1 adenoma) at week 20 and 2/3 rats (1 adenoma and 1 carcinoma) at week 24. Microscopically, all hepatocellular carcinomas were of trabecular pattern. There were no differences between the experimental groups in the histological patterns of hepatocellular carcinomas. Almost all of the liver cell neoplasms were GGT-positive, although a few neoplasms displayed an irregular GGT reaction. In other organs, no preneoplastic or neoplastic lesions were found.

## DISCUSSION

The results in the present study demonstrate that the induction of liver cell foci and neoplasms by FAA was inhibited by the plant phenol, EA, when it was orally given concurrently with the carcinogen. An inhibitory effect of EA on carcinogenesis in lung or skin has been reported by several investigators.[5-7, 16, 17] However, no studies on the modifying effect of EA on carcinogenesis in other organs including liver have been reported. In the above studies, EA was administered to mice by ip injection,[7] in the diet,[7] po,[7] in drinking water[6] or by topical application.[5, 16, 17] In the present study, EA was given to rats in the diet, and this was effective. The inhibitory effect of EA on the development of lung tumors was greater when it was administered by ip injection than in the diet.[7] Lesca[7] also reported the inhibitory effect of another plant phenol, chlorogenic acid, on pulmonary tumorigenesis. We have obtained similar results on liver carcinogenesis in rats.[18] Lesca[7] reported that ip injection of EA resulted in severe toxicity although dietary EA did not cause any toxicity. In the present study too, dietary administration of EA produced no toxic effects.

In the present study, we quantified the effect of EA on preneoplastic liver altered foci

induced by FAA. These lesions are considered to be precursors of liver cell neoplasms.[19] Similar results were obtained in previous studies using a similar experimental design, but with administration of an antioxidant, butylated hydroxytoluene.[20, 21] Thus, the inhibitory effect of chemicals on liver carcinogenesis can be detected at an early stage by quantification of foci.[21, 22]

Several potential mechanisms for the antimutagenic and anticarcinogenic effect of chemopreventive agents have been proposed[1, 12, 23, 24]: (a) by the formation of adducts with DNA, acting in a competitive manner to inhibit ultimate carcinogen:DNA adduct formation; (b) by the inhibition of the microsomal enzymes involved in the carcinogen formation; (c) by the stimulation of the detoxification enzymes. EA is also considered to exert its protective effect on mutagenesis and carcinogenesis by any one or more of the above mechanisms.[3, 4, 8, 9, 25-27] The liver and kidney showed the highest amounts of radioactivity after ip injections of [$^3$H]EA in mice.[9] Moreover, chronic administration of EA in drinking water enhanced the activity of glutathione S-transferase, a detoxifying enzyme, in mouse liver.[8] Thus, it seems likely that the protective effect of EA in the present study is due in major part to a change in the balance between enzyme activation and detoxification of the carcinogen. Since high concentrations of EA are present in a number of foods, such as fruits and vegetables, eaten by humans and appears to be well tolerated by both experimental animals[11] and humans,[28] this compound is considered to offer considerable promise as a chemopreventive agent in human carcinogenesis. However, its efficacy for chemoprevention of carcinogenesis and the precise mechanism of its action need to be investigated.

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