RESEARCH COMMUNICATION

# Inhibitory Effects of Dietary Monoglucosyl-rutin on Azoxymethane-induced Colon Carcinogenesis in Rats

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## Abstract

**The dietary effect of monoglucosyl-rutin (M-R), a flavonoid, on azoxymethane (AOM)-induced colon carcinogenesis was investigated in two experiments with 5 week old, F344 male rats. In the first experiment (5 weeks study), effects of MR on AOM (15 mg/kg body weight 3 times weekly)-induced formation of aberrant crypt foci (ACF) in five groups were assessed. In this experiment, group 3 given 500 ppm M-R with AOM had a significantly smaller number of ACF containing 4 or more aberrant crypts than group 1 with AOM alone, and groups 2 and 3 given 100 ppm or 500 ppm M-R respectively had significantly lower BrdU labeling indices in the epithelial cells of large bowel than group 1. For the second experiment, rats were divided into 8 groups. Groups 1-5 were given AOM as in the first experiment. Groups 2-5 were fed diets containing 100ppm or 500ppm M-R for 4 weeks in the initiation phase or 36 weeks in the post-initiation phase. Group 6 was given 500ppm M-R throughout the experiment, and group 7 was kept on the basal diet and served as a control. At the termination of the experiment (40 weeks after the start), groups 2-5 had significantly smaller numbers of positive cells with anti-proliferating cell nuclea antigen (PCNA) antibody than group 1. Furthermore, group 5 treated with 500ppm M-R for 36 weeks demonstrated tendencies for decrease in the incidence and multiplicity of colon tumors. These data suggest that M-R has the potential to inhibit AOM-induced colon carcinogenesis.**

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**Key words:** monoglucosyl-rutin, colon carcinogenesis, azoxymethane, rat

## Introduction

Colon cancer is one of the most common malignant neoplasms in the world. In Japan, it is the third leading cause of cancer death with a recent increase, which suggested to be related to a change in dietary habits (Broder, 1993; Haenszel et al., 1980; Weisburger, 1991). Dietary factors play an important role in human diseases including cancer. We have reported effects of different types naturally occurring or synthetic agents on carcinogen-induced various neoplasms in rodents (Mori et al., 1997; Mori et al., 1992b; Yoshimi et al., 1999).

Flavonoids are contained in vegetables and fruits and have a variety of biological actions as anti-inflammatory, anti-mutagenic, anti-carcinogenic, and free radical-scavenging agents (Husain et al., 1987; Robak and Gryglewski, 1988). It is said that flavonoids are possible chemopreventive or therapeutic agents against free radical-associated diseases such as a cancer (Stavric, 1994). Previous studies showed that they inhibit carcinogen-induced various neoplasms in rodents (Tanaka et al., 1997a; Tanaka et al., 1997b; Yang et al., 1997). Rutin, one example, is water-insoluble and is contained in buckwheat, which has been used as a food for maintaining health, green tea, or other edible plants like potato and tomato. It is known that rutin has a protective effects against carcinogen-induced DNA-damage (Deschner et al., 1991; Webster et al., 1996) and an inhibitory effect on AOM-induced colon carcinogenesis (Deschner et al., 1991). alphaG-

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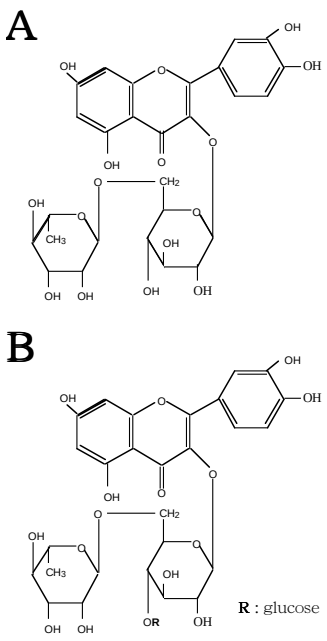


**Figure 1. Chemical Structures of Rutin(A) and $4^G$-alpha-glucopyranosylrutin**

Rutin formed by enzymatic transglycosylation (Suzuki & Suzuki, 1991) has been used as a water-soluble colorant for processed foods in Japan. aG-Rutin is composed of mainly $4^G$-alpha-D-glucopyranosylrutin and a small amount of isoquercitrin (Takaya, 1992) and is easily hydrolyzed to rutin and glucose in the body. The chemical structures of $4^G$-alpha-D-glucopyranosylrutin and rutin are shown in Fig. 1. It was shown that alphaG-Rutin has protective effects on ferric nitrilotriacetate and g-ray-induced oxidative damage in mice (Shimoi *et al*., 1997a; Shimoi *et al*., 1997b). It is thus suggested that alphaG-Rutin acts as an inhibitory agent against free radical-associated diseases such as various cancers. Meanwhile, monoglucosyl-rutin is composed of only $4^G$-alpha-D-glucopyranosylrutin. In this study, we examined modulating effects of M-R on AOM-induced colon carcinogenesis in rats.

## Materials and Methods

*Animals and chemicals*

Male F344 rats were obtained from Japan SLC, Inc. (Shizuoka). AOM was purchased from Sigma Chemical Co. (St Louis, MO) and M-R was supplied from Tokyo Sugar Refining Co., Ltd. (Tokyo), respectively. Basal diet (CE-2) was supplied by CLEA Japan, Inc. (Tokyo). All animals were housed in wire cages (3 or 4 rats/cage). They had free access to water and diet under controlled environmental conditions of humidity (50±10%), lighting (12 h light/dark cycle) and temperature (23±2°C).

*Experimental procedure*

In experiment 1 (see Fig 2), a total of 37 rats were randomized into 5 groups. At 6 weeks of age, rats of groups 1-3 received a s.c. injection of AOM , once a week for three weeks, at a dose of 15 mg/kg body weight. Starting a week before the first AOM injection, group 2 was given the diet containing 100ppm M-R for 5 weeks. Similarly, groups 3 and 4 were given the diet with 500ppm M-R. Group 5 was kept on the basal diet alone and served as a control. The experiment was terminated at 5 weeks after the start. All rats were killed and the colons were fixed in 10% buffered formalin and stained with a 0.2% methylene blue for the analysis of ACF.

In experiment 2, a total of 135 rats were divided into 7 groups. Rats of groups 1-5 received AOM as in experiment 1. Rats of groups 2 and 3 were given diet containing 100ppm and 500ppm M-R, respectively, starting at 5 weeks of age and continued until 2 weeks after the last injection of AOM. Groups 4 and 5 were fed diets containing 100ppm and 500ppm M-R, respectively, for 36 weeks, starting from a week after the last injection of AOM. Group 6 was fed 500ppm M-R alone throughout the experiment. Group 7 served as an untreated control. At the termination of the study (week 40), all rats were killed by decapitation. At autopsy,

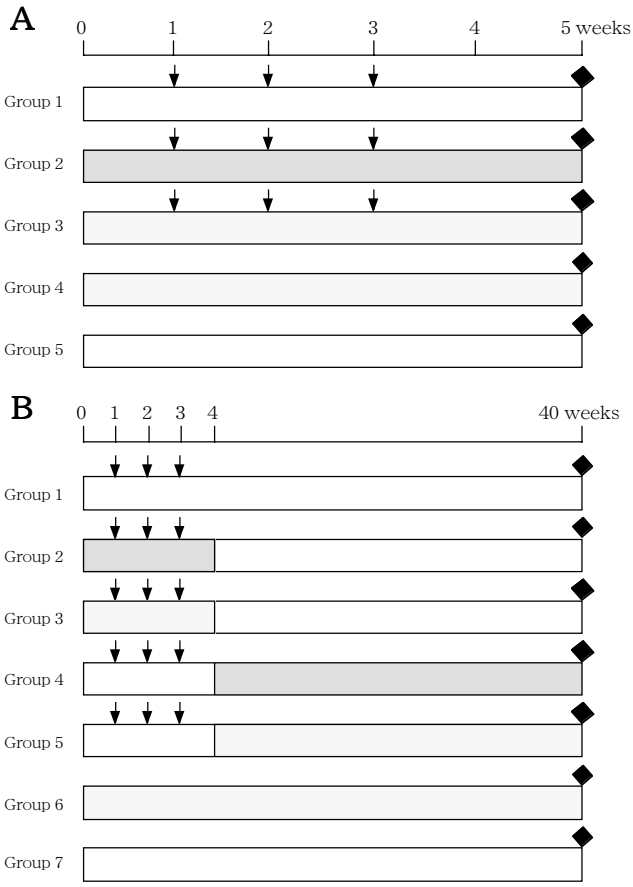


**Figure 2. Experimental protocol** (A) Experiment 1, (B) Experiment 2; azoxymethane (15 mg/kg body weight, s.c. injection) , 100ppm monoglucosyl-rutin in basal diet, 500ppm monoglucosyl-rutin in basal diet, basal diet, sacrifice

**Table 1. Body, Liver, and Relative Liver Weights of Rats at the Termination of the Experiment**

| Group no. | Treatment | No. of rats | Body weight (g) | Liver weight (g) | Relative liver weight (%) |
|---|---|---|---|---|---|
| 1 | AOM alone | 8 | 223 ± 8.78[a)] | 10.8 ± 1.13 | 4.83 ± 0.48 |
| 2 | AOM+100ppm Monoglucosyl-rutin | 9 | 215 ± 7.26 | 9.7 ± 0.67[b)] | 4.51 ± 0.24 |
| 3 | AOM+500ppm Monoglucosyl-rutin | 9 | 217 ± 6.80 | 10.0 ± 1.66 | 4.60 ± 0.68 |
| 4 | 500ppm Monoglucosyl-rutin alone | 5 | 230 ± 18.1 | 10.3 ± 1.30 | 4.47 ± 0.26 |
| 5 | No treatment | 5 | 228 ± 17.5 | 10.1 ± 1.24 | 4.41 ± 0.28 |

a) ; Mean ± S.D.
b) ; Significant difference from group 1 by Student's *t*- test ($P$<0.05)

**Table 2. Effects of Monoglucosyl-rutin on the Development of AOM-induced ACF**

| Group no. | Treatment | No. of rats | No. of ACF/colon | No. of aberrant crypts/colon | No. of aberrant crypts/focus | No.of ACF containing 4 or more crypts/colon | BrdU labeling index |
|---|---|---|---|---|---|---|---|
| 1 | AOM alone | 8 | 100 ± 27[a)] | 190 ± 63 | 1.87 ± 0.18 | 6.0 ± 5.1 | 11.00±1.71 |
| 2 | AOM+100ppm Monoglucosyl-rutin | 9 | 77 ± 29 | 138 ± 54 | 1.79 ± 0.08 | 3.3 ± 1.9 | 8.31± 0.87[b)] |
| 3 | AOM+500ppm Monoglucosyl-rutin | 9 | 81 ± 29 | 137 ± 55 | 1.68 ± 0.16[c)] | 1.3 ± 1.3[d)] | 7.82± 0.55[b)] |
| 4 | 500ppm Monoglucosyl-rutin alone | 5 | 0 | 0 | 0 | 0 | 7.77± 1.20 |
| 5 | No treatment | 5 | 0 | 0 | 0 | 0 | 7.87± 1.22 |

a) Mean ± S.D.
b) Significant difference from group 1 by Welch's method ($P$<0.05)
c) Significant difference from group 1 by Student's *t*-test ($P$<0.05
d) Significant difference from group 1 by Welch's method ($P$<0.05)

the intestines were excised, opened longitudinally, flushed clean with saline and examined for the presence of tumors. Abnormal lesions of other organs were also examined histologically. Colons, after fixation in 10% buffered formalin, were processed for histopathological examination by conventional methods. Intestinal neoplasms were diagnosed according to the criteria described by Ward (Ward, 1974).

*Determination of ACF*

The colons of all rats in the experiment 1 were used to score ACF. At autopsy, the colons were flushed with saline, excised, cut open longitudinally along the main axis and then washed with saline. The colons were cut into three sections (~4 cm each) starting from the anus, placed between filter papers to reduce mucosal folding and fixed in 10% buffered formalin for at least 24 h. Fixed colon sections were dipped in a 0.2% solution of methylene blue in distilled water for 30 s, then briefly washed with distilled water. Using a light microscope at a magnification of x40, ACF were distinguished by their increased size, their more prominent epithelial cells and their increased pericryptal space compared with surrounding normal crypts. The number of ACF observed per colon, the number of aberrant crypts observed in each focus and the location of each focus were recorded.

*BrdU and PCNA immunohistochemistry*

To assess the proliferative activity of colonic epithelium and the distribution of proliferative cells in the crypt, BrdU and PCNA immunohistochemistry (in experiments 1 and 2, respectively) was performed according to the methods described previously (Hirose et al., 1995). The labeled streptavidin biotin method was carried out according to the manufacturer's instructions using a LSAB KIT (DAKO). Primary antibody against BrdU (DAKO) was used at 1/100 dilution in 0.05 M Tris-HCl (pH 7.6) and incubation was performed for 2 h at room temprature. For PCNA, sections were incubated with primary antibodies against PCNA (1/100 dilution; Novocastra, Newcastle, UK) in 0.05 M Tris-HCl (pH 7.6), overnight at 4$^{o}$C in a humidified chamber. The

Table . Incidences, Multiplicity and PCNA Indices for Colon Tumors

| Group no. | Treatment | No. of rats | No. of rats bearing colon tumors(%) | Multiplicity | PCNA index |
|---|---|---|---|---|---|
| 1 | AOM alone | 23 | 15 (65) | 1.04±0.71 | 10.20±1.13[a)] |
| 2 | AOM+100ppmMonoglucosyl-rutin | 22 | 10 (45) | 0.86±0.94 | 7.94±1.07[b)] |
| 3 | AOM+500ppmMonoglucosyl-rutin | 24 | 13 (54) | 0.96±1.04 | 7.25±1.36[b)] |
| 4 | AOM→100ppmMonoglucosyl-rutin | 23 | 14 (61) | 0.87±0.81 | 7.02±1.43[b)] |
| 5 | AOM→500ppmMonoglucosyl-rutin | 25 | 10 (40) | 0.72±0.79 | 6.79±1.25[b)] |
| 6 | 500ppmMonoglucosyl-rutin alone | 10 | 0 (0) | 0 | 7.30±1.59 |
| 7 | No treatment | 10 | 0 (0) | 0 | 7.52±1.48 |

a) Mean ± S.D.
b) Significant difference from group 1 by Student's t-test (P<0.001)

numbers of BrdU and PCNA-positive nuclei in crypts per section were counted as described in previous papers (Mori *et al*., 1992a; Wang *et al*., 1993).

*Statistical analysis*

Fisher's exact probability test, Student's unpaired t-test or Welch's method were used for statistical analysis. A value of $P<0.05$ was considered significant.

## Results

In experiment 1, the liver weights of rats in group 2 were less than in group 1, but there were no significant differences between groups 1 and 3 (Table I). No differences of body weight, the relative liver weight and histopathological finding other than in the colon were recognized among the groups.

Data for the effects of M-R on the development of ACF are summarized in Table 2. Colonic ACF were noted only in rats treated with AOM (groups 1-3). The numbers of ACF/colon in groups 2 and 3 (77±29 and 81±29) and the numbers of aberrant crypts/colon in these groups (138±54 and 137±55) were respectively smaller than those of group 1 (100±27 and 190±63). However, no significant differences were apparent between the values of groups 2 or 3 and those of group 1. The numbers of aberrant crypts/focus in groups 1-3 were 1.87±0.18, 1.79±0.08, and 1.68±0.16, and the number of ACF containing four or more crypts/colon in these groups were 6.0±5.1, 3.3±1.9, and 1.3±1.3, respectively. Statistically, those in group 3 were significantly smaller than of group 1 ($P< 0.05$). Furthermore, the BrdU labeling indices of groups 2 and 3 (8.31±0.87 and 7.82±0.55) were smaller than in group 1 (11.0±1.71)($P< 0.05$).

In experiment 2, no differences in body weight, absolute and relative liver weights, or histopathological finding other than in the colon were recognized among the groups.

Data for the incidences and multiplicity of colonic neoplasms in each group are summarized in Table 3. There were no significant intergroup differences. Nevertheless, the values for groups 2 and 5 showed a tendency to be less than in group 1. The PCNA indices of the mucosal cells in the colonic epithelium of groups 2-5 (7.94±1.07, 7.25±1.36, 7.25±1.36, and 6.79±1.25, respectively) were significantly smaller than in group 1 (10.2±1.13)($P< 0.001$).

## Discussion

Monoglucosyl-rutin (M-R) is composed of only $4^G$-alpha-D-glucopyranosylrutin. Since the latter is formed by enzymatic transglycosylation(Suzuki & Suzuki, 1991) and is water-soluble, this chemical agent can to be absorbed in the bodies of rats, and is easily hydrolyzed to rutin and glucose. It is reported that rutin has an inhibitory effect on carcinogen-induced hyperproliferation of epithelial cells as well as on DNA-damage and occurrence of tumors in the colon of mice (Deschner *et al*., 1991).

Increasingly, ACF seen in colon of rodent and humans are regarded as preneoplastic lesions for colon cancer (McLellan & Bird, 1988; Pretlow *et al*., 1991) and therefore they have been proposed as an intermediate biomarkers for colon cancer. In the present study, exposure of 500 ppm M-R decreased the number of aberrant crypts/focus and ACF containing four or more crypts/colon. Furthermore, BrdU labeling indices in groups 2 and 3 were smaller than in group 1. These results indicate that M-R with anti-oxidative properties has a preventive effect on cell proliferation and development of preneoplastic lesions in colonic epithelium as well as against carcinogen-induced DNA-damage. Clear reasons for the fact that in experiment 1, liver weights in group 2 were smaller than in group 1 are not known.

In the present long-term study, unfortunately, exposure to M-R did not exert significant inhibitory effects in the development of colonic tumors due to AOM, although the groups given M-R and AOM had smaller PCNA indices of cells in the colonic epithelium than the group with AOM alone, and a tendency for decreased incidence and multiplicity of induced tumors. Furthermore, related flavonoids such as diosmin, hesperidin (Tanaka *et al*., 1997b), and morin (Tanaka *et al*., 1999a; Tanaka *et al*., 1999b) were earlier reported to exert protective effects and depress cell. Accordingly, it is expected that larger doses of M-R might inhibit tumor development in this carcinogenesis model.

M-R may be affected by microflora, since many flavonoids

have glucoside structures. It is reported that quercetin can be changed to the aglycone by b-glucosidase. Although quercetin glucoside is absorbed in the body more than guercetin aglycone, the anti-oxidative potential of quercetin aglycone is stronger than quercetin-glucoside (Hollman *et al*., 1995; Ioku *et al*., 1995). However, currently, it is not clear what kinds of flavonoids are actually affected by microflora in the colon.

In conclusion, we examined the modifying effects of dietary exposure of M-R, which is used as an anti-oxidant and a colorant for processed foods. Although obvious effects of this agent on tumors were not demonstrated, the results imply that M-R may have chemopreventive potential. Further studies are necessary to understand the biological activities of this flavonoid chemical.

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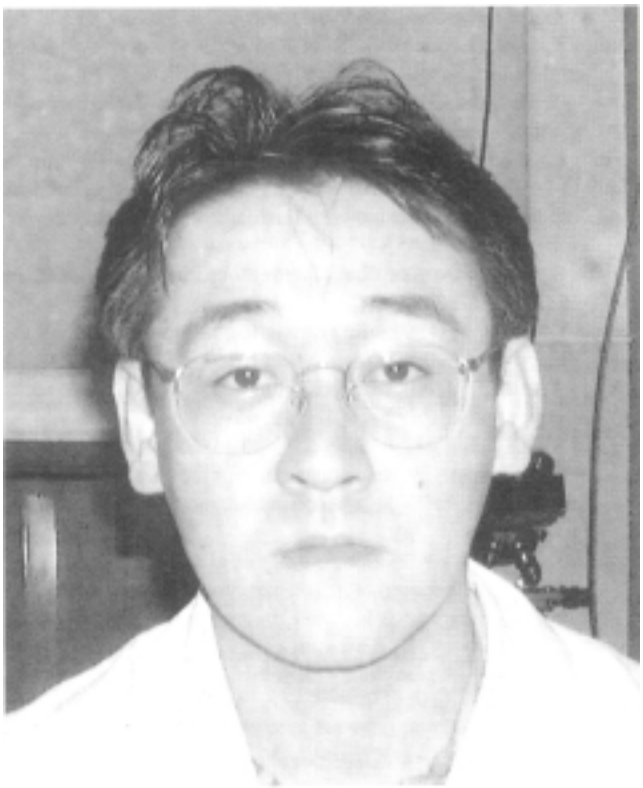

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