

CHEMOPREVENTIVE EFFECTS OF DIOSMIN AND HESPERIDIN ON *N*-BUTYL-*N*-(4-HYDROXYBUTYL)NITROSAMINE-INDUCED URINARY-BLADDER CARCINOGENESIS IN MALE ICR MICE

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The chemopreventive effects of 2 flavonoids (diosmin and hesperidin) on *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN)-induced urinary-bladder carcinogenesis were examined in male ICR mice. Animals were divided into 11 groups, and groups 1 to 7 were given OH-BBN (500 ppm) in the drinking water for 6 weeks. Groups 2 to 4 were fed diets containing the test compounds (group 2, 1000 ppm diosmin; group 3, 1000 ppm hesperidin; group 4, 900 ppm diosmin + 100 ppm hesperidin) for 8 weeks during the initiation phase, while groups 5 to 7 were fed these diets, respectively, for 24 weeks during the post-initiation phase. Groups 8 to 11 were controls, given only the test compounds or untreated basal diets throughout the experiment (weeks 1 to 32). The incidence of bladder lesions and cell-proliferation activity estimated by enumeration of silver-stained nucleolar-organizer-region-associated proteins (AgNORs) and by the 5-bromodeoxyuridine (BUdR)-labeling index was compared among the groups. Feeding of the test compounds, singly or in combination, during both phases caused a significant reduction in the frequency of bladder carcinoma and pre-neoplasia. Dietary administration of these compounds significantly decreased the AgNOR count and the BUdR-labeling index of various bladder lesions. These findings suggest that the flavonoids diosmin and hesperidin, individually and in combination, are effective in inhibiting chemical carcinogenesis of the bladder, and that such inhibition might be partly related to suppression of cell proliferation. *Int. J. Cancer* 73:719–724, 1997.

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The highest incidence of cancer of the urinary bladder is observed in the developed countries, with the exception of Japan and Russia. North America shows 3 times the incidence observed in Japan. (North America 29.2/100,000 vs. Japan 8.8/100,000) (Parkin *et al.*, 1993). North Africa is well known as a high-risk area for squamous-cell carcinoma of the bladder, certainly related to the presence of urinary schistosomiasis. Several epidemiological studies have related the geographical variation in the incidence of bladder cancer to exposure to known etiologic agents, including smoking, dietary sweeteners, *Schistosoma* infection and some industrial chemicals. However, the true etiology still remains unclear. Neoplasms in the urinary-tract epithelium possess several biological characteristics, such as multistage and multifocal carcinogenesis (Hicks, 1980). A high recurrence rate (50–70%) of superficial bladder tumors, even after curative transurethral resection, has often been reported (Green *et al.*, 1984). These successive, recurrent tumors may increase in their histological grade, and more than 15% of the patients suffer a progression to muscle-invasive disease, with subsequent poor prognosis (Kurth *et al.*, 1992). A number of anti-cancer drugs have been used, mainly via local instillation into the bladder, as an adjuvant to surgery, to suppress or prevent tumor recurrence, but only a few of these agents have been effective. Therefore, a new additional modality is required to achieve more satisfactory clinical control for this malignancy.

The term “cancer chemoprevention” refers to intervention with natural or non-toxic synthetic compounds, to prevent or delay the development of pre-malignant disease. Large amounts of epidemiological data have supported the inverse relation between the consumption of fruits and vegetables and the incidence of cancer

(Block *et al.*, 1992). Certain synthetic compounds (Kelloff *et al.*, 1992), such as oltipraz, piroxicam, indomethacin and D,L- α -difluoromethylornithin have been reported to have inhibitory effects on experimentally induced bladder-cancer models. Some natural compounds also inhibit chemically induced bladder carcinogenesis (Hirose *et al.*, 1995; Tanaka *et al.*, 1993). The flavonoids are considered to be a rich source of chemopreventive agents, since they have various therapeutic biological activities (Middleton and Kandaswami, 1994). They are known to have anti-inflammatory, anti-allergic, anti-viral, anti-mutagenesis and anti-proliferative properties. A major flavonoid, quercetin, which is an anti-oxidant and scavenger of free radicals, has been reported to inhibit chemical carcinogenesis in various organs (Tanaka, 1994). In addition, some flavonoids may affect drug-metabolizing enzyme activities, such as xanthine-oxidase activity and cellular protein phosphorylation (Middleton and Kandaswami, 1994). A flavanone, hesperidin (3',5,7-trihydroxy-4-methoxyflavanone 7-rhamnoglucoside) (Fig. 1a) and a flavone, diosmin (3',5,7-trihydroxy-4'-methoxyflavone 7-rutinoside) (Fig. 1b) are known to be contained in edible plants, fruits and vegetables, and are especially abundant in the peel of citrus fruits. These compounds are known to increase capillary resistance in various conditions, and a micronized complex of 90% diosmin + 10% hesperidin (Daflon, 500 mg) has been used clinically in France from 1971 for the treatment of human diseases such as chronic venous insufficiency and acute hemorrhoids (Berqvist *et al.*, 1981). Hesperidin possesses significant anti-inflammatory and analgesic effects (Galati *et al.*, 1994), which might be related to the inhibition of prostaglandin biosynthesis. Diosmin has been shown to have a protective effect against oxygen radicals *in vivo* and *in vitro* (Lonchampt *et al.*, 1989), and improves the inflammatory reaction by affecting the synthesis of prostaglandins and thromboxane. These compounds and their metabolites (hesperitin and diosmetin) have anti-mutagenic activities (Edenharder *et al.*, 1993). Our group has recently reported on the chemopreventive effects of some flavonoids, including diosmin, hesperidin and chalcone, on chemically induced rat colon, esophageal and oral cancers (Makita *et al.*, 1996; Tanaka *et al.*, 1997a,b).

In the present study, we investigated the possible chemopreventive effects of the dietary administration of diosmin and hesperidin, alone and in combination (diosmin:hesperidin = 9:1), during the initiation and post-initiation stages, on *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (OH-BBN)-induced bladder carcinogenesis in male ICR mice. In addition, to examine the effects of these compounds on cell-proliferation activity in the bladder transitional epithelium and to clarify the underlying mechanism, we measured the 5-bromodeoxyuridine (BUdR)-labeling indices and the number of silver-stained-nucleolar-organizer-region-associated proteins (AgNOR).

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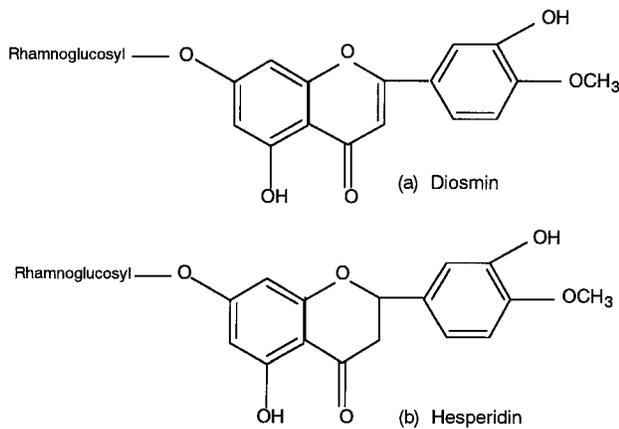


FIGURE 1 – Chemical structures of the test compounds: diosmin (a) and hesperidin (b).

MATERIAL AND METHODS

Animals, diets and carcinogen

A total of 231 male ICR mice, 5 weeks old, were purchased from Japan SLC (Hamamatsu City, Japan). All animals were housed in plastic cages with wood chips in an air-conditioned room at $23 \pm 2^\circ\text{C}$ (SD), $50 \pm 10\%$ relative humidity, under a 12-hr light/dark cycle. OH-BBN was obtained from Tokyo Chemical Industry, Tokyo, Japan. Powdered CE-2 (CLEA Japan, Tokyo, Japan) was used as a basal diet throughout the experiment. Diosmin and hesperidin were purchased from Sigma (St. Louis, MO) and Fluka (Buchs, Switzerland), respectively. Experimental foods mixed with the test compounds were prepared each week. OH-BBN was given to the mice in tap water at a concentration of 500 ppm. The drinking water including carcinogen was prepared every other day. All the animals were allowed free access to food and water.

Experimental procedure

The mice were randomly divided into 11 groups (Fig. 2). Beginning at 6 weeks of age, the mice in groups 1 through 7 were given OH-BBN (500 ppm) in the drinking water for 6 weeks. Group 2 was given food containing 1000 ppm diosmin [1 mg (1.643 μmol)/g diet], group 3, that containing 1000 ppm hesperidin [1 mg (1.638 μmol)/g diet] and group 4, that containing both compounds (900 ppm diosmin and 100 ppm hesperidin), starting at 5 weeks of age until 1 week after cessation of the carcinogen exposure. Groups 2, 3 and 4 were then given the basal diet, maintained for 24 weeks. Group 5 was fed food mixed with diosmin, group 6, that with hesperidin and group 7, that with both chemicals, starting at 1 week after cessation of the OH-BBN treatment; these 3 groups continued on these diets for 24 weeks. Groups 8 to 10 were not exposed to the carcinogen, and were fed food containing only the test compounds for the duration of the experiment. Group 11 was given the basal diet and tap water throughout the experiment and served as an untreated control group (Fig. 2).

All mice were carefully observed, and daily consumption of the OH-BBN-containing water and the foods mixed with the test compounds was recorded. The experiment was terminated at 32 weeks, and all the animals were killed under ether anesthesia. At autopsy, the urinary bladders of all the mice were inflated with 10% buffered formalin, fixed overnight in 10% buffered formalin, bisected longitudinally, inspected for gross lesions, then embedded in paraffin for histopathological evaluation on hematoxylin-and-eosin-stained sections. Other organs were also examined histopathologically. Urinary-bladder epithelial lesions, including hyperplasia, pre-cancerous lesion (dysplasia), papilloma and carcinoma were diagnosed according to the criteria described by Fukushima *et al.* (1982).

Determination of cell-proliferation activity by AgNOR enumeration and BUdR labeling indices

To assess the proliferative activity of the urinary-bladder lesions, the number of AgNOR per nucleus and the BUdR-labeling indices were quantified according to the methods described (Tanaka *et al.*, 1994). For measurement of the BUdR-incorporating nuclei, the animals were given an i.p. injection of 50 mg/kg body weight BUdR (Sigma) 1 hr prior to killing. The urinary bladder was removed and fixed in 10% buffered formalin for histopathology, the AgNOR count and the BUdR-labeling indices. Three serial sections (3 μm thick) were made after embedding in paraffin. On one section, AgNOR staining was carried out by a 1-step silver colloid method. The AgNOR were visualized as distinct black dots. The number of discrete dots and dot aggregate were counted on 100 nuclei of various lesions and non-lesional areas using a microscope with $\times 400$ magnification. AgNOR that were aggregated and inseparable or clustered were considered as one structure. The highest number of AgNOR dots that were visible within a nucleus by focusing the microscope through the nucleus was counted. The mean number of AgNOR per nucleus was then calculated for each specimen. Another section was used for the detection of BUdR incorporation by means of an immunohistochemical analysis kit (Amersham, UK). The labeling indices of BUdR (%) were calculated by counting the labeled nuclei of 100 cells from each lesion under $\times 400$ magnification. The remaining section was used for histopathological diagnosis.

Statistical analysis

Statistical analysis of the incidence of lesions was performed using Fisher's exact probability test or Chi-squared test, and the data from the measurements of body weight, liver weight, AgNOR enumeration and BUdR-labeling index were compared, using Student's *t*-test for unpaired samples or a 2-sample *t*-test with Welch's correction. The results were considered statistically significant if the *p* value was 0.05 or less.

RESULTS

General observations

All mice in groups 1 to 10 tolerated well the oral administration of OH-BBN, diosmin and/or hesperidin. There were no significant differences in the mean intake of OH-BBN or food among groups 1 to 7 (OH-BBN, 0.214–0.230 mg/day/mouse). The mean intake of food was constant and similar in the different groups during the whole treatment period (4.75–5.25 g/day/mouse). The mean body and liver weights at the end of the study are indicated in Table I. The mean body weight of the mice in group 1 (OH-BBN alone) was significantly lower than that of group 11 (untreated control) ($p < 0.005$). The mean body weights of groups 2 (OH-BBN + diosmin) and 3 (OH-BBN + hesperidin) were significantly greater than that of group 1 ($p < 0.005$ and $p < 0.01$ respectively). The mean liver weight of group 1 was significantly lower than that of group 11 ($p < 0.05$). The average liver weights of groups 2, 3, 4 (OH-BBN + diosmin and hesperidin) and 7 (OH-BBN \rightarrow diosmin and hesperidin) were significantly greater than that of group 1 ($p < 0.001$, $p < 0.02$, $p < 0.005$ and $p < 0.02$ respectively). The average relative liver weights (g/100 g body weight) of groups 2, 4 and 7 were significantly greater than that of group 1 ($p < 0.05$, $p < 0.02$ and $p < 0.05$ respectively).

Incidence of tumors and pre-cancerous lesions

Hyperplasia, pre-cancerous lesion (dysplasia), transitional-cell papilloma, and transitional-cell carcinoma developed in the urinary bladders of the mice in groups 1 through 7. The incidence of these in each group are shown in Table II. In group 1 (OH-BBN alone), the incidence of transitional-cell papilloma and transitional-cell carcinoma was 19% (5/26 mice) and 62% (16/26 mice) respectively. On the other hand, only a few of the mice given the test

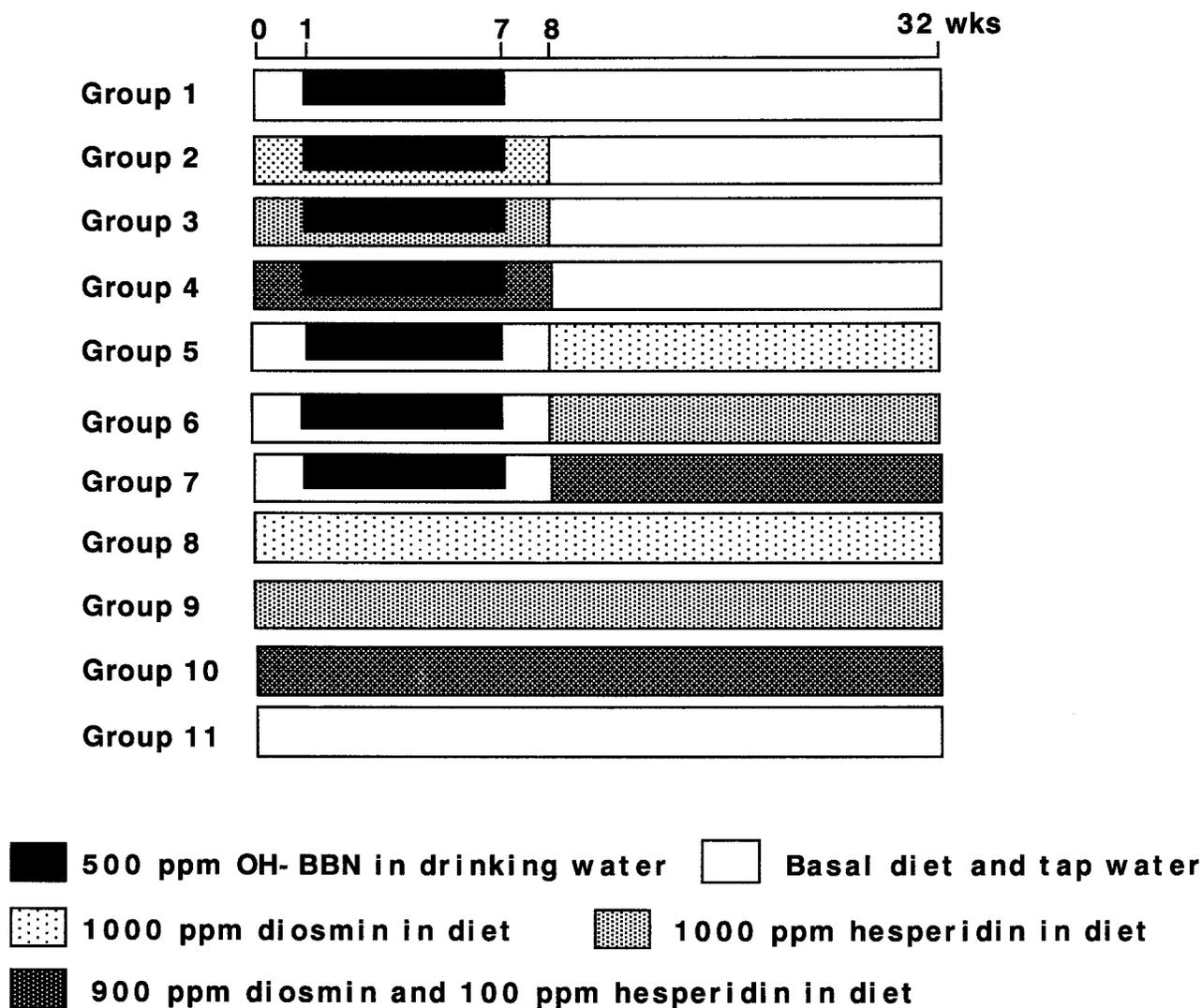


FIGURE 2 – Experimental protocol.

TABLE I – BODY, LIVER AND RELATIVE LIVER WEIGHTS OF MICE IN EACH GROUP

Group number	Treatment	Number of mice examined	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	OH-BBN	26	49.7 ± 4.6 ^{1,2}	2.9 ± 0.4 ³	5.8 ± 0.5
2	OH-BBN + diosmin (initiation phase)	24	53.1 ± 3.2 ⁴	3.3 ± 0.3 ⁵	6.1 ± 0.5 ⁶
3	OH-BBN + hesperidin (initiation phase)	23	53.9 ± 5.4 ⁷	3.2 ± 0.4 ⁸	5.9 ± 0.4
4	OH-BBN + combination (initiation phase)	25	51.4 ± 5.0	3.2 ± 0.3 ⁹	6.2 ± 0.6
5	OH-BBN → diosmin (post-initiation phase)	24	51.7 ± 5.0	2.9 ± 0.4	5.6 ± 0.6
6	OH-BBN → hesperidin (post-initiation phase)	25	51.4 ± 3.3	2.9 ± 0.4	5.7 ± 0.5
7	OH-BBN → combination (post-initiation phase)	24	51.6 ± 3.5	3.2 ± 0.4 ⁸	6.2 ± 0.7 ⁶
8	Diosmin	15	51.0 ± 7.2	3.0 ± 0.6	6.0 ± 1.2
9	Hesperidin	15	56.1 ± 7.4	3.2 ± 0.4	5.7 ± 0.6
10	Combination	15	55.9 ± 7.5	3.3 ± 0.4	6.0 ± 0.9
11	No treatment	15	55.0 ± 6.0	3.2 ± 0.5	5.9 ± 0.8

¹Mean ± SD.—^{2,3}Significantly different from group 11 by Student's *t*-test (²*p* < 0.005; ³*p* < 0.05).—⁴Significantly different from group 1 by 2-sample *t*-test with Welch's correction (*p* < 0.005).—⁵⁻⁹Significantly different from group 1 by Student's *t*-test (⁵*p* < 0.001; ⁶*p* < 0.05; ⁷*p* < 0.01; ⁸*p* < 0.02; ⁹*p* < 0.005).—Combination: 900 ppm diosmin and 100 ppm hesperidin in diet.

compounds during the OH-BBN administration, or of those fed the test compounds after OH-BBN exposure, presented transitional-cell carcinomas (21% in group 2, 13% in group 3, 24% in group 4, 8% in groups 5, 20% in group 6 and 21% in group 7). These incidence rates were significantly smaller than those of group 1

(*p* < 0.005, *p* < 0.001, *p* < 0.01 and *p* < 0.0001). No such neoplasms developed in the animals of groups 8 to 11.

As for transitional-cell hyperplasia (simple hyperplasia and papillary or nodular hyperplasia), the incidence in group 1 was 96% with simple hyperplasia, and 50% with papillary or nodular

TABLE II – INCIDENCE OF PATHOLOGICAL LESIONS IN THE URINARY BLADDER

Group number	Treatment	Number of mice examined	Number of mice with various pathological lesions (%)				
			SH	PN	DYS	PAP	TCC
1	OH-BBN	26	25 (96)	13 (50)	24 (92)	5 (19)	16 (62)
2	OH-BBN + diosmin	24	23 (96)	6 (25)	14 (58) ¹	4 (17)	5 (21) ²
3	OH-BBN + hesperidin	23	22 (96)	9 (39)	15 (65) ³	1 (4)	3 (13) ⁴
4	OH-BBN + combination	25	21 (84)	8 (38)	13 (52) ²	3 (12)	6 (24) ⁵
5	OH-BBN → diosmin	24	18 (75) ³	5 (21) ³	10 (42) ⁶	3 (13)	2 (8) ⁷
6	OH-BBN → hesperidin	25	18 (72) ³	6 (24)	10 (40) ⁷	3 (12)	5 (20) ²
7	OH-BBN → combination	24	15 (63) ²	4 (17) ⁸	10 (42) ⁶	3 (13)	5 (21) ²
8	Diosmin	15	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
9	Hesperidin	15	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
10	Combination	15	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
11	No treatment	15	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

SH, simple hyperplasia; PN, papillary or nodular hyperplasia; DYS, dysplasia; PAP, papilloma; TCC, transitional-cell carcinoma.

^{1-4,6-8}Significantly different from group 1 by Fischer's exact probability test (¹ $p < 0.01$; ² $p < 0.005$; ³ $p < 0.05$; ⁴ $p < 0.001$; ⁶ $p < 0.0005$; ⁷ $p < 0.0001$; ⁸ $p < 0.02$).–⁵Significantly different from group 1 by the Chi-squared test ($p < 0.01$).–Combination: 900 ppm diosmin and 100 ppm hesperidin in diet.

TABLE III – AGNOR COUNT PER NUCLEUS OF NON-LESIONAL TRANSITIONAL EPITHELIUM AND VARIOUS LESIONS IN THE URINARY BLADDER

Group number	Treatment	Normal	Non-lesional area	Hyperplasia		DYS	PAP	TCC
				SH	PN			
1	OH-BBN	ND	2.59 ± 0.22 ¹	3.06 ± 0.30	3.29 ± 0.34	3.62 ± 0.38	4.26 ± 0.35	4.73 ± 0.47
2	OH-BBN + diosmin	ND	2.24 ± 0.11 ²	2.55 ± 0.13 ³	3.11 ± 0.25	3.24 ± 0.15 ⁶	3.92 ± 0.13	4.25 ± 0.35 ²
3	OH-BBN + hesperidin	ND	2.25 ± 0.13 ⁵	2.68 ± 0.23 ²	3.36 ± 0.19	3.38 ± 0.22	3.46 ± 0.59 ²	4.10 ± 0.45 ⁶
4	OH-BBN + combination	ND	2.35 ± 0.19 ²	2.70 ± 0.22 ²	2.99 ± 0.35	3.11 ± 0.20 ⁵	3.25 ± 0.29 ⁷	3.93 ± 0.29 ⁷
5	OH-BBN → diosmin	ND	2.39 ± 0.19	2.74 ± 0.15 ²	2.94 ± 0.15 ²	3.14 ± 0.21 ³	3.69 ± 0.19 ⁵	4.15 ± 0.24 ³
6	OH-BBN → hesperidin	ND	2.34 ± 0.21 ²	2.63 ± 0.24 ³	2.91 ± 0.20	3.11 ± 0.18 ³	3.44 ± 0.15 ³	3.91 ± 0.53 ⁷
7	OH-BBN → combination	ND	2.28 ± 0.18 ²	2.46 ± 0.16 ⁵	2.76 ± 0.25 ²	2.96 ± 0.19 ³	3.16 ± 0.08 ³	3.42 ± 0.41 ⁷
8	Diosmin	2.41 ± 0.20	ND	ND	ND	ND	ND	ND
9	Hesperidin	2.67 ± 0.15	ND	ND	ND	ND	ND	ND
10	Combination	2.52 ± 0.15	ND	ND	ND	ND	ND	ND
11	No treatment	2.52 ± 0.23	ND	ND	ND	ND	ND	ND

SH, simple hyperplasia; PN, papillary or nodular hyperplasia; DYS, dysplasia; PAP, papilloma; TCC, transitional-cell carcinoma; ND, not determined.

¹Mean ± SD.–^{2,5-7}Significantly different from group 1 by Student's *t*-test (² $p < 0.05$; ⁵ $p < 0.005$; ⁶ $p < 0.01$; ⁷ $p < 0.001$).–^{3,4}Significantly different from group 1 by 2-sample *t*-test with Welch's correction (³ $p < 0.001$; ⁴ $p < 0.01$).–Combination: 900 ppm diosmin and 100 ppm hesperidin in diet.

TABLE IV – BUdR-LABELING INDEX (%) OF NON-LESIONAL TRANSITIONAL EPITHELIUM AND VARIOUS LESIONS IN THE URINARY BLADDER

Group number	Treatment	Normal	Non-lesional area	Hyperplasia		DYS	PAP	TCC
				SH	PN			
1	OH-BBN	ND	0.57 ± 1.13 ¹	2.50 ± 2.97	1.00 ± 0.71	6.50 ± 2.35	3.80 ± 1.64	25.46 ± 6.92
2	OH-BBN + diosmin	ND	0.22 ± 0.44	1.20 ± 1.47	1.56 ± 1.13	5.08 ± 1.93	3.33 ± 1.53	17.78 ± 9.20 ²
3	OH-BBN + hesperidin	ND	0.20 ± 0.45	0.46 ± 0.69 ³	0.67 ± 1.16	4.40 ± 2.30	3.00 ± 0.00	9.20 ± 3.11 ⁴
4	OH-BBN + combination	ND	0.13 ± 0.35	0.40 ± 0.74 ³	1.80 ± 1.30	4.80 ± 3.49	4.00 ± 2.31	14.20 ± 4.44 ⁵
5	OH-BBN → diosmin	ND	0.33 ± 0.52	0.56 ± 1.01	0.25 ± 0.50	3.33 ± 2.83 ²	2.00 ± 1.87	9.20 ± 4.55 ⁶
6	OH-BBN → hesperidin	ND	0.20 ± 0.45	0.33 ± 0.71 ³	0.50 ± 0.58	3.40 ± 2.61	2.40 ± 1.82	12.71 ± 6.90 ⁶
7	OH-BBN → combination	ND	0.29 ± 0.49	0.50 ± 0.76 ³	0.75 ± 0.89	3.67 ± 1.97 ²	1.43 ± 0.79 ⁵	9.88 ± 2.64 ⁷
8	Diosmin	0.40 ± 0.74	ND	ND	ND	ND	ND	ND
9	Hesperidin	0.27 ± 0.59	ND	ND	ND	ND	ND	ND
10	Combination	0.47 ± 0.74	ND	ND	ND	ND	ND	ND
11	No treatment	0.33 ± 0.62	ND	ND	ND	ND	ND	ND

SH, simple hyperplasia; PN, papillary or nodular hyperplasia; DYS, dysplasia; PAP, papilloma; TCC, transitional-cell carcinoma; ND, not determined.

¹Mean ± SD.–^{2,4-6}Significantly different from group 1 by Student's *t*-test (² $p < 0.05$; ⁴ $p < 0.001$; ⁵ $p < 0.01$; ⁶ $p < 0.005$).–^{3,7}Significantly different from group 1 by 2-sample *t*-test with Welch's correction (³ $p < 0.05$ and ⁷ $p < 0.001$).–Combination: 900 ppm diosmin and 100 ppm hesperidin in diet.

hyperplasia. The incidences of simple hyperplasia in groups 5 to 7 and those of papillary or nodular hyperplasia in groups 5 and 7 were significantly smaller than that of group 1 ($p < 0.05$, $p < 0.005$ and $p < 0.02$). The incidence of pre-cancerous lesion (dysplasia) of group 1 was 92%. The frequency of dysplasia in groups 2 through 7 (40–65%) was significantly lower than that of group 1 ($p < 0.01$, $p < 0.05$, $p < 0.005$, $p < 0.0005$ and $p < 0.0001$).

AgNOR enumeration and BUdR-labeling indices

The results of the morphometric analysis of AgNOR and the BUdR-labeling indices are summarized in Tables III and IV. The mean AgNOR counts and the BUdR-labeling indices in the non-lesional epithelium and in various lesions of the group exposed to OH-BBN alone (group 1) was greater than in the normal transitional epithelium of the untreated control group (group 11).

The numbers of AgNOR nucleus in the non-lesional transitional epithelium of groups 2, 3, 4, 6, 7 were significantly lower than that of group 1 ($p < 0.05$, $p < 0.005$). As for simple hyperplasia, the AgNORs/nucleus counts in groups 2 to 7 and the BUdR-labeling indices in groups 3, 4, 6 and 7 were significantly smaller than that in group 1 ($p < 0.05$, $p < 0.005$ and $p < 0.001$). The AgNOR counts for papillary or nodular hyperplasia in groups 5 and 7 were significantly smaller than that in group 1 ($p < 0.05$), but the differences of the BUdR-labeling indices on this lesion in groups 2 to 7 and in group 1 were not significant. In dysplasia, the AgNOR counts in group 2 and groups 4 to 7, and the BUdR-labeling indices in groups 5 and 7, were significantly lower than those in group 1 ($p < 0.01$, $p < 0.005$, $p < 0.001$ and $p < 0.05$). The AgNOR counts for transitional-cell papilloma in groups 3- to 7 and the BUdR-labeling indices for transitional-cell papilloma in group 7 were significantly lower than in group 1 ($p < 0.05$, $p < 0.001$, $p < 0.005$ and $p < 0.01$). Both transitional-cell carcinoma values in groups 2 to 7 were significantly lower than those in group 1 ($p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.005$).

DISCUSSION

Our results demonstrate that dietary diosmin and hesperidin, alone or in combination, during the initiation or the post-initiation phase, effectively inhibited mouse bladder carcinogenesis induced with OH-BBN. The reduction rate of "initiation" feeding was hesperidin > diosmin > combination (diosmin + hesperidin), and that of "post-initiation" feeding was diosmin > hesperidin > combination. The combination of diosmin with a low level of hesperidin did not show a distinct synergetic effect on the tumor incidence. In this study, both test compounds inhibited cell-proliferation activity of the bladder neoplasms. These results are in line with our earlier studies using other carcinogenesis models (Makita *et al.*, 1996; Tanaka *et al.*, 1997a,b). Otherwise, we found no effect of the tested compounds on the BUdR-labelling index and the AgNOR counts in normal tissue. The tested compounds showed a chemopreventive effect only in pre-cancerous lesion (dysplasia) and carcinoma, and no toxicity in normal tissue. However, due to the AgNOR-number results, some additional effects appear to inhibit cell proliferation by the combination of hesperidin and diosmin. The AgNOR count in the groups treated with the combination regimen was smaller for almost all the lesions than that of the single-regimen-treated groups. The number and size of AgNOR are known to alter in 2 conditions: increased cellular differentiation to specialized function in the absence of proliferation, and increased cellular production in G₁ of components required for cell division. Our earlier study indicated a step-wise increase of AgNOR number from histologically normal transitional epithelium through hyperplasia and pre-cancerous lesion (dysplasia) to transitional-cell carcinoma in OH-BBN-induced urinary-bladder carcinogenesis (Takeuchi *et al.*, 1990). Some flavonoids have been reported to induce morphological differentiation in rat neural cells (data not shown). The combination regimen (hesperidin and diosmin) may decrease cell proliferation by the induction of cell differentiation; its effect was remarkable as compared with that of the single regimen.

Several mechanisms could be considered to exert anti-carcinogenic effects by diosmin and hesperidin in bladder carcino-

genesis, because of the various therapeutic biological activities of these compounds (Middleton and Kandaswami, 1994). They have anti-oxidative and free radical scavenger activities with various degrees. Free radicals are possibly related to both the initiation and the promotion phases of carcinogenesis (Cerutti, 1985). The effect of anti-oxidants in decreasing such free-radical damage is considered to contribute to lowering the incidence of cancer (Tanaka, 1994). In the present study, the dietary administration of diosmin and hesperidin either in the initiation or in the post-initiation phase significantly reduced the incidence of pre-cancerous lesion (dysplasia) and transitional-cell carcinoma. These results are comparable to other chemopreventive studies with several natural anti-oxidants (Tanaka *et al.*, 1994). Hesperidin was more chemopreventive than diosmin when fed during the initiation stage. This may be explained by the differences of ring responsibility for anti-oxidant activity in flavonoids (Jovanovic *et al.*, 1996). Cell proliferation is thought to play an important role (Tanaka, 1994) in multistage carcinogenesis, including bladder tumorigenesis. The results of the present study indicate that the dietary administration of these compounds could effectively inhibit 2 stages of bladder tumorigenesis induced by OH-BBN. Dietary administration of these compounds either in the initiation or in the post-initiation phase significantly reduced cell-proliferation activity in the bladder epithelium with or without pre-cancerous lesion (dysplasia) and carcinoma. Some of our other studies have demonstrated the protective properties of xanthophylls (Tanaka *et al.*, 1993) and protocatechuic acid (Hirose *et al.*, 1995) on bladder tumorigenesis in rodents, and the capacity of these substances to prevent carcinogenesis is considered to be partly due to the suppression of cell proliferation as revealed by AgNOR enumeration. Non-steroidal anti-inflammatory agents (NSAID) can also suppress urinary-bladder carcinogenesis (Kelloff *et al.*, 1992). Rao *et al.* (1996) have demonstrated that the NSAID, aspirin, ketoprofen and sulindac, potent inhibitors of the arachidonic acid pathway, are effective inhibitors of OH-BBN-induced bladder carcinogenesis in mice. Since diosmin and hesperidin have anti-inflammatory and analgesic effects related to the inhibition of prostaglandin biosynthesis, this mechanism may also contribute to their chemopreventive effect on bladder tumorigenesis. OH-BBN-induced bladder carcinogenesis is caused by a major urinary metabolite of OH-BBN, *N*-butyl-*N*-(3-carboxypropyl)nitrosamine, which is formed from OH-BBN after ω -oxidation in the liver by a drug-metabolizing enzyme. Flavonoids could affect drug-metabolizing enzyme activity, such as xanthine-oxidase activity and cellular protein phosphorylation (Middleton and Kandaswami, 1994). Diosmetin and diosmin might be conjugated by a specific isoform of UDP-glucuronyltransferase by the use of liver microsomal preparations. This function may also explain the anti-tumor protective properties of diosmin and hesperidin. The biological activities of other flavonoids have been reported, but the exact mechanism of cancer chemoprevention by diosmin and hesperidin remains to be elucidated.

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