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Effect of hesperetin, a citrus flavonoid, on bacterial enzymes and carcinogen-induced aberrant crypt foci in colon cancer rats: a dose-dependent study

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Abstract

Hesperetin, an important bioactive compound in Chinese traditional medicine, has antioxidant and anticarcinogenic properties. Hesperetin is found in abundance in orange and grape juices (200–590 mg L^{-1}) consumed in the daily diet. We have investigated the effect of different doses of hesperetin on faecal and colonic mucosal bacterial enzymes and aberrant crypt foci (ACF) in 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in male Wistar rats. The rats were divided into six groups and were fed a modified pellet diet for 16 weeks. Group 1 served as control and group 2 received the modified pellet diet along with hesperetin (30 mg kg^{-1}). The rats in groups 3–6 rats were given a weekly subcutaneous injection of DMH (20 mg kg^{-1}) for the first four weeks. Hesperetin was supplemented orally at different doses (10, 20 or 30 mg kg^{-1}) for a total of 16 weeks. At the end of the experimental period all rats were killed. In DMH-treated rats, the activity of faecal and colonic mucosal bacterial enzymes, such as β-glucuronidase, β-galactosidase, β-glucosidase, nitroreductase, sulfatase and mucinase, were significantly elevated, but in rats supplemented hesperetin along with DMH the activity was significantly lowered ($P < 0.05$). The total number of aberrant crypts was significantly increased in unsupplemented DMH-treated rats, while hesperetin supplementation to DMH-treated rats significantly reduced the total number of crypts. The results demonstrated that hesperetin supplementation at a dose of 20 mg kg^{-1} played a potent role in suppressing the formation of aberrant crypt foci and reducing the activity of bacterial enzymes in colon cancer.

Introduction

Colon cancer is still a leading cause of cancer death in the United States and is increasing at an alarming rate in Asia (Greenlee et al 2000). In India, there are approximately 3.5 million cases of cancer, of which approximately 35 000 are found to be colorectal cancer (Shrikhande et al 2007). Colon cancer is associated with environmental factors and diet, particularly in terms of animal protein and combined fat. The suspected effect of diet on colon carcinogenesis is mediated through the changes in the composition of intestinal microflora as well as the compounds secreted into the gut (Wollowski et al 2001).

The chemically-induced colon cancer rat model is widely used to investigate the prevention and pathogenesis of this disease. Colon cancer can be induced in experimental animals with 1,2-dimethylhydrazine (DMH), as a procarcinogen. DMH yields azoxymethane, which is hydroxylated to an active agent by the intestinal flora (Agner et al 2005). The intestinal bacterial enzymes are important in the metabolism of procarcinogens and production of tumour promoters in the colon, and hence they play a significant role in the pathogenesis of colon cancer (Ohkami et al 1995). The changes in the activity of faecal bacterial and mucosal enzymes represent an actual increase or decrease in the enzyme activity or simply a non-specific response to changes in colonic dry matter or water content. However, the alterations can also be due to colonic and faecal protein, which can be of dietary origin, host secretions or bacterial protein. Therefore, the protein content can vary independently from bacterial counts and bacterial activity (Mykkanen et al 1998). The carcinogenic effect of endogenous toxic and genotoxic compounds to act as carcinogens are possibly influenced by the activity of bacterial enzymes such as nitroreductase, β-glucuronidase, and β-glucosidase (Rowland 1991). The relationship between diet and rat faecal bacterial enzymes is implicated in colon cancer. Faecal mutagenicity appears to be dependent on diet; people on a typical 'Western' diet are at higher

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risk for colon cancer and have higher faecal mutagenicity than those on a vegetarian diet (de Kok & van Maanen 2000).

Aberrant crypt foci (ACF), which are morphologically abnormal crypts in the colonic mucosa, are reliable intermediate biomarkers for colon carcinogenesis (Bird 1995). Based on the proposition that these lesions are early preneoplastic events of colorectal cancer, ACF are being used as a short-term bioassay to identify modulators of colon carcinogenesis (Hirose et al 2003).

Hesperetin (5,7,3′trihydroxyl-4′-methoxyl-flavanone) is a bioactive compound, belonging to the class of flavonoids called flavanones, which are abundant in citrus fruits. A daily intake of citrus juices, such as orange and grape juice, contains approximately 200–590 mg L^{-1} hesperetin. Daily ingestion of citrus flavonoids has been estimated to be approximately 68 g on average in the USA, mainly ingested via fruit juices (Scholz et al 2007). Hesperetin is used in Chinese traditional medicine as it possesses several biological and pharmacological properties, including antioxidant (Cai et al 2004) and anti-carcinogenic properties (Cooray et al 2004). It is also specifically known to alter the signal transduction pathways (O'Prey et al 2003). The effect of hesperetin on faecal bacterial enzymes in relation to experimental colon carcinogenesis has not been studied so far. Thus, our aim was to investigate the short-term effects of hesperetin on ACF, and faecal and mucosal bacterial enzyme activity during DMH-induced colon carcinogenesis.

Materials and Methods

Animals and diet

Male albino Wistar rats (4-weeks old, 120–150 g) were obtained from the Central Animal House, Rajah Muthiah Medical College and Hospital (RMMCH), Annamalai University. The rats were kept in polypropylene cages with paddy husk for bedding and were fed a standard pellet diet for one-week acclimatization. Thereafter, the rats were randomly allocated into six groups of 10 animals and were kept in a room with controlled temperature (24 ± 2°C), humidity (50 ± 10%), and lighting (12-h light/dark periods). The rats were cared for in compliance with the principles and guidelines of the Ethical Committee for Animal Care of Annamalai University in accordance with the Indian National Law on Animal Care and Use (Reg. No. 190/2007/CPCSEA). A commercial pellet diet containing 4.2% fat (Hindustan Lever Ltd, Mumbai, India) was powdered and mixed with 15.8% peanut oil making a total of 20% fat, which was the modified pellet diet freely fed to all the rats (Table 1). The total caloric intake of the rats in all the groups was adjusted to be the same. Body weights were recorded once a week for the entire 16-week study.

Tumour induction

From the first week of administering the diet, animals (4-weeks old) were given subcutaneous injections of DMH (20 mg kg^{-1}) (Sigma Chemical, USA), once a week for the first four weeks.

Table 1 Composition of the diet

	Commercial pellet diet 84.2%	Peanut oil 15.8%	Total %
Protein	17.7	–	17.7
Fat	4.2	15.8	20.0
Carbohydrates	50.5	–	50.5
Fibre	3.4	–	3.4
Minerals	6.7	–	6.7
Vitamins	1.7	–	1.7

Hesperetin preparation

Hesperetin was purchased from Sigma Chemical Co. (USA). Hesperetin was suspended in carboxymethyl cellulose at a concentration of 0.1% at three different doses of 10, 20 and 30 mg kg^{-1}. Hesperetin was administered orally by intragastric intubation every day.

Experimental design

Rats were randomly distributed into six groups of 10 animals each, as follows: group 1 received modified pellet diet and served as control; group 2 received modified diet with hesperetin (30 mg kg^{-1}) everyday throughout the experimental period; groups 3–6 received modified diet with DMH (20 mg kg^{-1}) once a week, subcutaneously for the first four weeks. In addition, groups 4–6 received 10, 20 or 30 mg kg^{-1} hesperetin in 0.1% carboxymethyl cellulose orally every day, respectively, for a total of 16 weeks. At the end of the experimental period, food was withheld overnight and the rats were killed by decapitation. Figure 1 depicts the schematic representation of the experimental design.

Determination of aberrant crypt foci (ACF)

At the end of the 16-week study, rat colons were removed and flushed with potassium phosphate-buffered saline (0.1 M, pH 7.2). Colons were split open longitudinally and placed on strips of filter paper with their luminal surface open and exposed. Another strip of filter paper was placed on top of the luminal surface. The colons were then secured and fixed in a tray containing 10% buffered formalin overnight. Each fixed colon was cut into proximal and distal portions of equal lengths and each portion was cut further into 2-cm long segments. Each segment was placed in a Petri dish and stained with 0.2% methylene blue solution for 2 min. The segments were then transferred to another Petri dish containing buffer to wash off excess stain. The segments were examined using a light microscope at low magnification to score the total number of ACF as well as the number of crypts per focus. ACF were distinguished from normal crypts by their thicker, darker-stained, raised walls with elongated slit-like lumens and significantly increased distance from the lamina to basal surface of cells. ACFs in the colon were counted as described by Bird (1987).

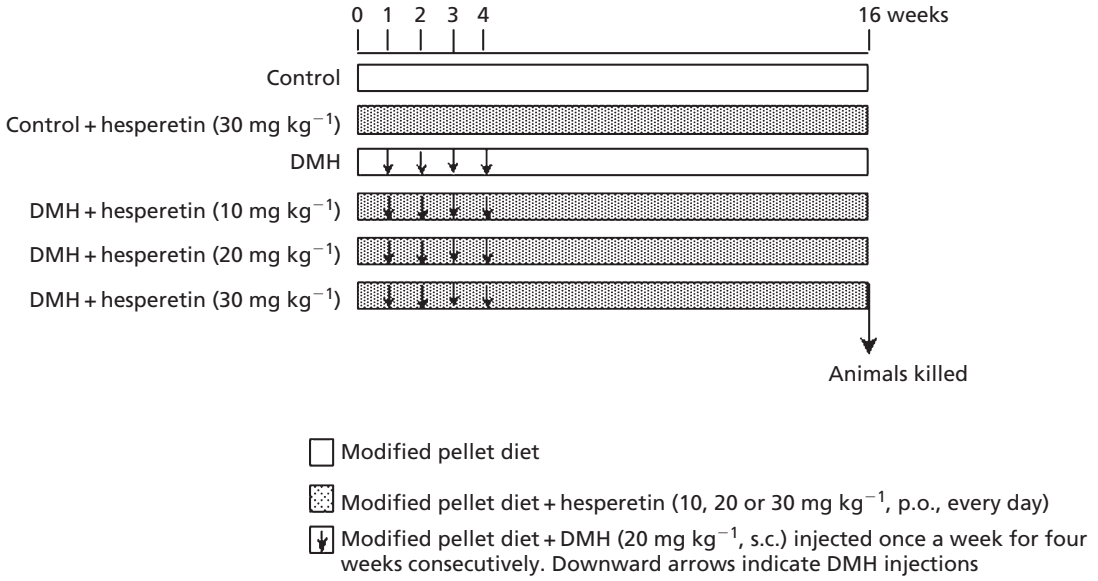

Figure 1 Schematic representation of the experimental design.

Faecal and mucosal tissue processing

Fresh faecal pellets were collected for the assay of faecal bacterial enzymes. The mucosa from the colon was collected by scraping with a slide. The faecal pellets and colonic mucosa were homogenized using phosphate-buffered saline, centrifuged at 2000 *g* for 10 min at 4°C, and the supernatant collected for the assay of the activity of faecal and colonic mucosal bacterial enzymes.

Measuring enzyme activity

β-Glucosidase activity

β-Glucosidase activity was measured by the method of Freeman (1986). *β*-Glucosidase assay mixture contained 3 μM *p*-nitrophenol-*β*-D-glucopyranoside (substrate), 50 mM potassium phosphate buffer (pH 7.2), a suitable amount of the faecal or mucosal suspension and the final volume was made up to 1 mL. After incubation at 37°C for 60 min, the reaction was arrested by the addition of 0.2 M Na_2CO_3. The enzyme activity was determined spectrophotometrically measuring the absorption produced by *p*-nitrophenol at 450 nm. For faecal samples values were expressed as mg *p*-nitrophenol liberated min^{-1} (g protein)$^{-1}$, whilst for mucosal samples values were expressed as μg *p*-nitrophenol liberated h^{-1} (g protein^{-1}).

β-Glucuronidase activity

β-Glucuronidase activity was measured by the method of Freeman (1986). *β*-Glucuronidase assay mixture contained 0.02 M phosphate-buffered saline, 0.1 M EDTA, 0.01 M *p*-nitrophenyl-*β*-D-glucuronide (substrate), a suitable amount of faecal or mucosal suspension and the final volume was made up to 1 mL. The mixture was incubated at 37°C for 15 min and the reaction was arrested by the addition of 0.2 M glycine buffer. The amount of *p*-nitrophenol released was determined spectrophotometrically at 540 nm by comparison with a standard nitrophenol curve.

β-Galactosidase activity

β-Galactosidase activity was measured by the method of Freeman (1986). The assay mixture contained 3 mM *p*-nitrophenol-*β*-D-galactopyranoside (substrate), a suitable amount of faecal or mucosal suspension and the final volume was made up to 1 mL. The assay mixture was incubated at 37°C for 15 min and the reaction was arrested by the addition of 0.25 M Na_2CO_3. The enzyme activity was measured spectrophotometrically at 405 nm.

Mucinase activity

Mucinase activity was measured by the method of Shiau & Chang (1983). The assay mixture contained 0.2 M porcine gastric mucin and a known amount of faecal suspension. The mixture was incubated at 37°C for 25 min. The amount of reducing sugar released was measured by the Nelson-Somoyogi method (Nelson 1944) at 620 nm. Values are expressed as mg glucose liberated min^{-1} (mg protein)$^{-1}$.

Nitroreductase activity

Nitroreductase activity was measured by the method of Bratton & Marshal (1939). The mixture contained 1.5 mM *p*-nitrobenzoic acid, 2.0 mL phosphate buffer and 2.0 mL faecal suspension. The reaction was arrested by the addition of trichloroacetic acid and centrifuged. The amount of *p*-aminobenzoic acid transformed was measured at 550 nm.

Sulfatase activity

Sulfatase activity was measured by the method of Rowland et al (1983). The assay mixture contained 0.02 M phosphate-buffered saline, 1 mM *p*-nitrocatechol sulfate and 1 mM EDTA. The reaction was arrested by the addition of 0.01 M NaOH and the amount of *p*-nitrocatechol liberated was read at 492 nm spectrophotometrically. Values were expressed as μmol *p*-nitrocatechol liberated min^{-1} (g protein)$^{-1}$.

Protein content of suspensions

Protein content of faecal or mucosal suspension was determined by the method of Lowry et al (1951) using bovine serum albumin as standard at 660 nm.

Statistical analysis

Values are given as means ± s.d. Data were analysed by one-way analysis of variance and any significant difference among treatment groups was evaluated by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant at $P < 0.05$. All statistical analyses were made using SPSS11.0 software package (SPSS, Tokyo, Japan).

Results

Effect of hesperetin and DMH on body weight and growth rate

Body weight and growth rate in all the groups increased at a normal rate (Figure 2). From week 0 to 16, variable changes were observed in the body weight and growth rate in the rats administered different doses of hesperetin (10, 20 or 30 mg kg^{-1}). The average body weight of DMH-treated rats maintained on the high fat diet showed a significantly ($P < 0.05$) lower gain in body weight and growth rate throughout the experimental period as compared with groups 1 and 2. On administration of different doses of hesperetin (groups 4, 5 and 6), the body weight and growth rate were significantly elevated as compared with the un-supplemented DMH-treated rats. Moreover, group 5 rats showed a significantly ($P < 0.05$) improved weight gain as compared with group 3.

Effect of hesperetin and DMH on tumour incidence

Incidence of tumours was significantly higher in the DMH-treated rats (Table 2). For the rats administered hesperetin at different doses (groups 4, 5 and 6) the incidence of tumour was significantly reduced. The tumour incidence in rats treated with DMH along with hesperetin 20 mg kg^{-1} was markedly lower in group 5 as compared with groups 4 and 6. Tumour number and size in the tumour-bearing rats were not significantly different among groups 4, 5 and 6.

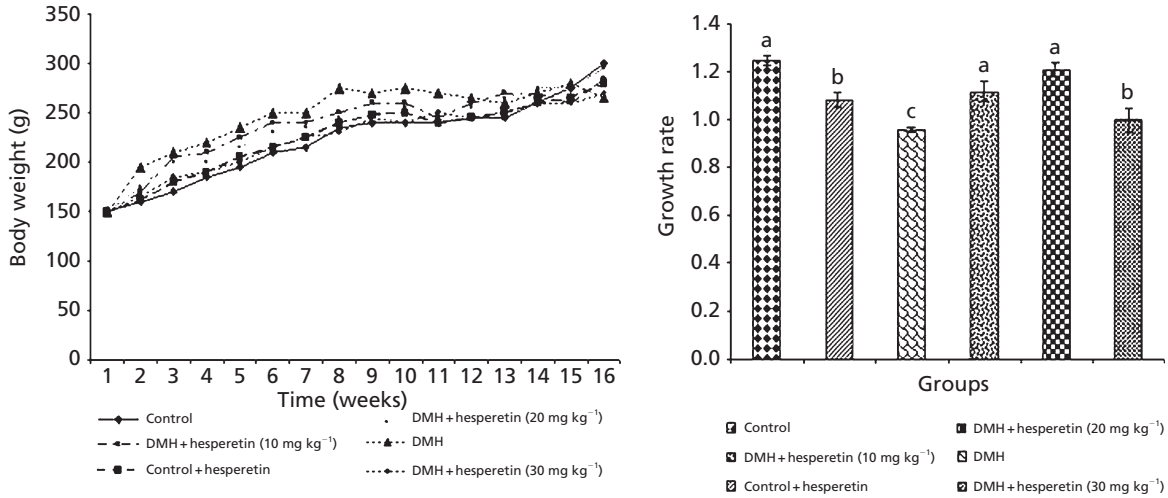

Figure 2 Effect of hesperetin and DMH on body weight and growth rate of control and experimental colon cancer rats. Data are presented as the means ± s.d. of each group. $^{a-c}P < 0.05$, values not sharing a common superscript letter are significantly different.

Table 2 Effect of hesperetin on colonic tumour incidence and the number of tumours per tumour-bearing rat

Groups	No. of rats	No. of tumours/polyps-bearing rats	Tumour/polyps incidence* (%)	Total tumours/ polyps number	No. of tumours (polyps)/tumour-bearing rat
Control	10	0	0	Nil	Nil
Control + hesperetin (30 mg kg^{-1})	10	0	0	Nil	Nil
DMH	10	10^{a}	100^{a}	19^{a}	1.9^{a}
DMH + hesperetin (10 mg kg^{-1})	10	7^{b}	70^{b}	10^{b}	1.4^{b}
DMH + hesperetin (20 mg kg^{-1})	10	5^{c}	50^{c}	6^{c}	1.2^{c}
DMH + hesperetin (30 mg kg^{-1})	10	6^{d}	60^{d}	8^{c}	1.3^{d}

*(Number of tumour-bearing rats/total number of rats in each groups) × 100. $^{a-d}P < 0.05$, value not sharing a common superscript letter are significantly different.

Effect of hesperetin and DMH on aberrant crypts and number of crypts/focus

ACF were stereoscopically distinct from normal crypts as evidenced by their darker staining, larger size, elliptical shape, thicker epithelial lining, and larger pericryptal zone, shown in Figure 3, and total number of crypts, shown in Table 3. No ACFs were detected in the control rats or in group 2. DMH-treated rats (group 3) had a significantly higher number of total aberrant crypts as compared with the control rats. Administration of hesperetin at different doses (10, 20 or 30 mg kg^{-1} for groups 4, 5 and 6, respectively) significantly reduced the total number of aberrant crypts. There was a total reduction of 75% in ACF incidence in rats administered a dose of 20 mg kg^{-1} hesperetin, while the reduction rate was 52% and 60% respectively in groups 4 and 6. Thus, a more pronounced effect on ACF reduction was noted in rats supplemented with hesperetin 20 mg kg^{-1} (group 5).

Effect of hesperetin and DMH on faecal bacterial enzymes

Table 4 summarizes the activity of the bacterial enzymes β-glucosidase, β-glucuronidase, β-galactosidase, nitroreductase, mucinase and sulfatase obtained from fresh faecal

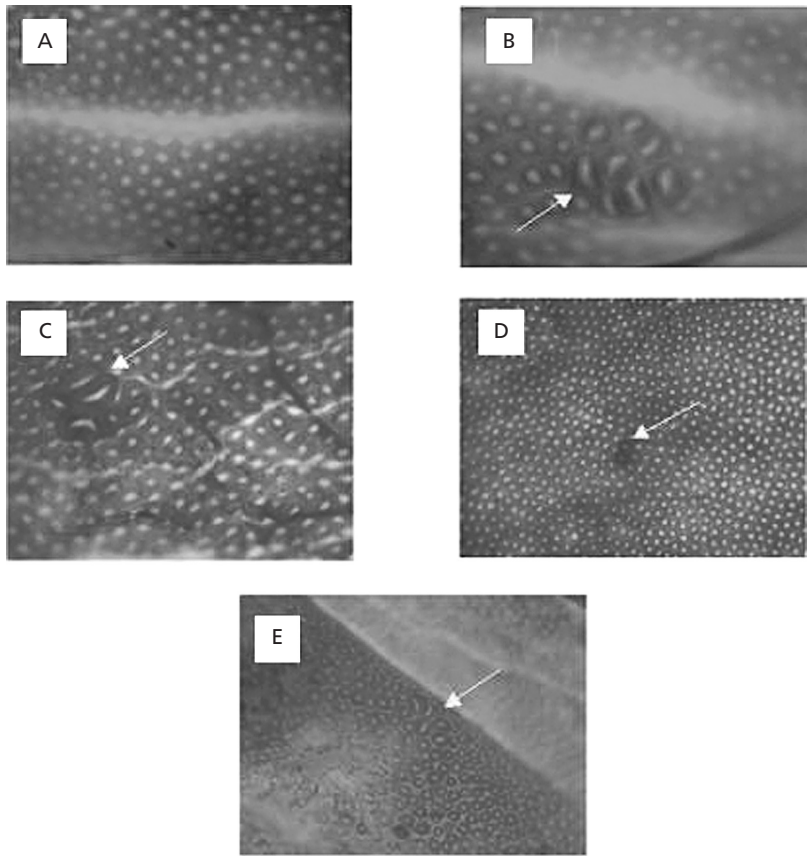

Figure 3 Effect of hesperetin on ACF formation and crypt multiplicity. A. Control colonic tissue. B. DMH-treated rats. C. DMH + hesperetin (10 mg kg^{-1}). D. DMH + hesperetin (20 mg kg^{-1}). E. DMH + hesperetin (30 mg kg^{-1}).

Table 3 The effect of hesperetin on number of aberrant crypt foci with different crypt multiplicity on DMH-induced colon cancer

Groups	Total no. of ACF	No. of foci containing			% Inhibition of ACF
		1 crypt	2 crypts	>4 crypts	
Control	Nil	Nil	Nil	Nil	–
Control + hesperetin (30 mg kg^{-1})	Nil	Nil	Nil	Nil	–
DMH (n = 5)	110.3 ± 10.7[a]	37.8 ± 3.4[a]	25.7 ± 2.1[a]	46.8 ± 4.5[a]	–
DMH + 10 mg kg^{-1} hesperetin (n = 5)	60.8 ± 5.7[b]	22.6 ± 2.1[b]	17.3 ± 1.6[b]	20.9 ± 2.0[b]	52
DMH + 20 mg kg^{-1} hesperetin (n = 5)	34.9 ± 3.2[c]	19.4 ± 1.6[b]	15.5 ± 1.4[b]	–	75
DMH + 30 mg kg^{-1} hesperetin (n = 5)	45.3 ± 4.3[d]	20.1 ± 1.8[b]	17.9 ± 1.6[b]	7.1 ± 0.6[c]	65

Data are presented as the means ± s.d. of each group. [a–d]$P < 0.001$ the values not sharing a common superscript letter are significantly different.

Table 4 Effect of hesperetin on faecal bacterial enzymes of control and experimental colon cancer rats

Groups	β-Glucuronidase[¶]	β-Glucosidase[¶]	β-Galactosidase[¶]	Mucinase[♣]	Sulfatase[#]	Nitroreductase[§]
Control	17.8 ± 1.7^{a}	70.7 ± 6.8^{a}	30.6 ± 2.9^{a}	10.7 ± 1.0^{a}	18.0 ± 1.7^{a}	46.8 ± 4.5^{a}
Control + hesperetin	19.1 ± 1.8^{ab}	100.1 ± 10.6^{b}	41.4 ± 4.0^{b}	9.7 ± 0.9^{b}	19.7 ± 1.9^{b}	41.9 ± 4.0^{b}
DMH	21.3 ± 2.0^{bc}	117.0 ± 11.2^{c}	45.1 ± 4.3^{c}	13.8 ± 1.3^{c}	28.1 ± 2.7^{c}	111.2 ± 10.7^{c}
DMH + hesperetin (10 mg kg^{-1})	19.6 ± 1.8^{ab}	98.3 ± 9.6^{b}	42.2 ± 4.0^{b}	12.6 ± 1.2^{d}	22.9 ± 2.2^{d}	91.3 ± 8.9^{b}
DMH + hesperetin (20 mg kg^{-1})	18.2 ± 1.62^{a}	73.0 ± 7.1^{a}	31.2 ± 2.9^{a}	11.2 ± 0.99^{a}	16.2 ± 1.4^{a}	50.4 ± 4.9^{a}
DMH + hesperetin (30 mg kg^{-1})	20.8 ± 1.8^{c}	62.4 ± 6.1^{d}	36.6 ± 3.2^{d}	12.4 ± 1.1^{d}	13.6 ± 1.1^{e}	92.9 ± 9.1^{d}

[¶]μg *p*-Nitrophenol liberated h^{-1} (g protein)$^{-1}$. [§]μmol *p*-Aminobenzoic acid liberated min^{-1} (g protein)$^{-1}$. [#]μmol *p*-Nitrocatechol liberated min^{-1} (g protein)$^{-1}$. [♣]mg Glucose liberated min^{-1} (mg protein)$^{-1}$. Data are presented as the means ± s.d. of each group. [a–e]$P < 0.05$ the values not sharing a common superscript letter are significantly different.

samples of control and experimental rats. The specific activity of these faecal bacterial enzymes was significantly ($P < 0.05$) higher in the DMH-treated rats (group 3) as compared with the control rats. Treatment with different doses of hesperetin (10, 20 or 30 mg kg^{-1}) along with DMH significantly decreased activity of these enzymes, the activity being more pronounced in animals supplemented with 20 mg kg^{-1} hesperetin (group 5).

Effect of hesperetin and DMH on colonic mucosal bacterial enzymes

Table 5 shows the enzyme activity of mucosal bacterial enzymes β-glucosidase, β-glucuronidase, β-galactosidase and nitroreductase from control and experimental rats. The activity of these enzymes was significantly elevated in DMH-treated rats as compared with the control rats at the end of 16 weeks. Supplementation with hesperetin at different doses (groups 4, 5 and 6) significantly decreased the activity of the mucosal enzymes, and a more pronounced effect was observed in the rats supplemented with 20 mg kg^{-1} hesperetin (group 5).

Discussion

We have evaluated the dose-dependent inhibitory effects of hesperetin (a flavonone) on colon carcinogenesis induced by DMH. Hesperetin 20 mg kg^{-1} (group 5) had a more pronounced anticancer effect as compared with the 10 or 30 mg kg^{-1} doses.

ACF are readily discernible 'preadenomatous' morphologically putative lesions within the colonic mucosa of rodents that may contribute to the stepwise progression to colon cancer. The formation and growth of ACF are associated with the induction of colon tumours in rats and are influenced by exposure to chemopreventive agents (Kanna et al 2003). It has been proven that the number of crypts/focus increases with time after carcinogen treatment and that the ACF demonstrate increased cell proliferation in rodents (Mori et al 2004). Onoue et al (1997) suggested that reduction of bacterial enzyme activity was paralleled by a decrease in the frequency of colonic ACF. Thus, one of the plausible explanations for the reduction in tumour incidence and ACF development may be associated with the reduced activity of faecal and colonic mucosal enzymes. Sengottuvelan et al (2006) have already reported a higher number of total aberrant crypts in DMH-treated rats. Similarly, in this study DMH-treated rats showed an increased number and size of crypts, among which 80% of ACF in rats treated with DMH alone contained less than four crypts/focus. In this context, ACF containing four or more crypts is known to correspond to the promotion stage of colon carcinogenesis (Wei et al 2003). Hesperetin administration at different doses (10, 20 or 30 mg kg^{-1}) virtually reduced the development of ACF and we could observe ACF containing not more than one or two aberrant crypts. Moreover, the dose of 20 mg kg^{-1} (group 5) had a greater impact on the suppression of the formation of the preneoplastic lesions in DMH-induced colon carcinogenesis (Figure 3). Our results correlated with Miyagi et al (2000), who had shown that consumption of orange juice reduced ACF and tumour incidence in azoxymethane-induced

Table 5 Effect of hesperetin on colonic mucosal bacterial enzymes of control and experimental colon cancer rats

Groups	β-Glucuronidase[¶]	β-Glucosidase[¶]	β-Galactosidase[¶]	Nitroreductase[§]
Control	5.4 ± 0.5^{a}	9.9 ± 0.96^{a}	22.2 ± 2.1^{a}	17.7 ± 1.7^{ab}
Control + hesperetin	3.3 ± 0.38^{b}	$12.1 \pm 1.4b^{e}$	27.6 ± 3.2^{b}	19.8 ± 2.3^{b}
DMH	7.6 ± 0.74^{c}	18.3 ± 1.7^{c}	43.8 ± 4.2^{c}	29.2 ± 2.8^{c}
DMH + hesperetin (10 mg kg^{-1})	7.3 ± 0.71^{cd}	15.6 ± 1.5^{d}	38.3 ± 3.6^{d}	23.0 ± 2.2^{d}
DMH+ hesperetin (20 mg kg^{-1})	4.4 ± 0.43^{a}	10.8 ± 0.95^{ab}	23.3 ± 2.0^{ae}	18.4 ± 1.6^{ab}
DMH + hesperetin (30 mg kg^{-1})	6.9 ± 0.60^{d}	12.9 ± 1.1^{e}	26.6 ± 2.3^{be}	16.8 ± 1.4^{a}

[¶]μg *p*-Nitrophenol liberated h^{-1} (g protein)$^{-1}$. [§]μmol *p*-Aminobenzoic acid liberated min^{-1} (g protein)$^{-1}$. Data are presented as the means ± s.d. of each group. [a–e]$P < 0.05$ the values not sharing a common superscript letter are significantly different.

colon cancer in rats due to the presence of hesperidin (glyco form of hesperetin).

The intestinal flora comprises a metabolically active group of organisms that produce enzymes catalysing the metabolism of procarcinogens and formation of tumour promoters, which in turn play a significant role in the pathogenesis of colon cancer (Ohkami et al 1995). Moreover, a high-fat diet is known to be a risk factor, which alters bacterial enzymes and is responsible for the conversion of procarcinogens to proximal carcinogens (Jacobs et al 1981). The bacterial enzymes assayed in this study were potential mediators of colon carcinogenesis (Rowland et al 1998). Bacterial β-glucuronidase is believed to be largely responsible for the hydrolysis of glucuronide conjugates in the gut. Increase in β-glucuronidase may increase the hydrolysis of carcinogen–glucuronide conjugate, liberating carcinogen and/or co-carcinogen within the colonic lumen (Devasena & Menon 2003). The increased production of short chain fatty acids inhibit the bacterial degradation of primary to secondary bile acids, which are known to promote colon cancer in carcinogen-treated rats (Lee & Lee 2000). Deeptha et al (2006) had shown an increased activity of β-glucuronidase in DMH-treated rats. Similarly, this study has shown an increased activity of β-glucuronidase in DMH-treated rats, while hesperetin supplementation at different doses significantly reduced the activity. Pronounced lowering of the β-glucuronidase activity was seen in rats supplemented with hesperetin 20 mg kg^{-1} (group 5).

Bacterial β-glucosidase hydrolyses DMH to its toxic metabolite, methylazoxymethanol (Ohkami et al 1995). The luminal carcinogens derived from the dietary β-glucosides might be reduced by dietary fibres. The bacterial β-glucosidase is potentially important in the generation of toxicants and carcinogens in the colon. The activity of β-galactosidase is a marker of the fermentative capacity of the intestinal microflora. Suppression of these microfloral enzymes may explain the lower incidence of colon cancer induced by the chemical carcinogen, DMH, in rats fed hesperetin. Our results indicated the elevated levels of β-glucosidase and β-galactosidase in DMH-treated rats, while hesperetin supplementation reduced the activity of these enzymes.

Colonic permeability may be an important factor in the aetiology of colon cancer. Chemicals probably need to reach the colonic mucosa before they can induce tumour formation, and they may do so by penetrating the lumen (Shiau & Chang 1983). Mucinase is also a hydrolytic enzyme secreted by the intestinal microflora, which degrades the protective mucus layer of the colon. Mucins form gels coating the intestinal mucosa, functioning as lubricants and probably as a chemical or mechanical barrier against bacteria (Nalini et al 2004). A change in mucinase activity is accompanied by a change in the rate of mucin degradation and a shift in the balance between mucin secretion and degradation (Shiau & Ong 1992). Mucinase activity was increased in the colon contents of animals given DMH as compared with control rats. In animals given DMH plus hesperetin, mucinase activity was significantly reduced as compared with animals given DMH only. Thus, hesperetin at different doses was found to inhibit faecal mucinase activity. Significant reduction ($P < 0.05$) in the mucinase activity on hesperetin supplementation may have helped to maintain the gel-coating barrier, protecting against bacteria and toxins in the intestinal lumen. These results could explain the lower number and incidence of colonic tumours in DMH-treated rats supplemented with hesperetin.

Sulfatase and nitroreductase are the key enzymes responsible for the metabolic activation of many procarcinogens and xenobiotics (Goldin 1986). Faecal sulfatase activity helps in the desulfation of conjugated toxins and in the degradation of sulfated mucins. Nitroreductase reduces heterocyclic and aromatic nitro compounds, which are extensively used in industry and medicine, to produce carcinogenic derivatives (Lee & Lee 2000). Our data showed increased activity of sulfatase and nitroreductase in unsupplemented DMH-treated rats. Administration of hesperetin decreased the sulfatase and nitroreductase activity in DMH-treated groups, which could be related to the changes in intestinal microflora and their metabolic activity. Alterations of microbial enzymes could contribute to changes in intestinal metabolism of hesperetin and thus may have an impact on the magnitude and type of response that occurs after chronic exposure. In general, elevated enzyme activity indicates an increased potential for bioactivation of xenobiotics (Chau et al 2005).

Control rats were also treated with hesperetin (30 mg kg^{-1}) to examine the role of hesperetin itself under controlled conditions and to evaluate statistically the extent of benefit it offers in DMH-induced colon cancer. The data did not show any significant effect on faecal/colonic mucosal enzymes and the formation of ACF when hesperetin was administered.

Conclusions

Supplementation of hesperetin (10, 20 or 30 mg kg^{-1}) may have exerted a favourable effect on improving the intestinal milieu and health, mainly by reducing the activity of undesirable bacterial enzymes in faeces as well as the exposure of intestinal lumen to the harmful compounds formed along the intestinal tract. Our results suggested that hesperetin could be exploited as a potential functional ingredient and offer opportunities to develop new formulations of functional foods. Overall, it seemed that intestinal bacteria played a key role in the activation and detoxification of DMH, which has been an area of research long ignored. Further experiments, including preclinical efficacy and study on the mechanisms of action are warranted to fully evaluate hesperetin for its anticancer properties and to understand its mode of action.

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