Carcinogenesis vol.21 no.10 pp.1909–1915, 2000

Red meat and colon cancer: dietary haem, but not fat, has cytotoxic and hyperproliferative effects on rat colonic epithelium

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High intake of red meat is associated with an increased risk of colon cancer. It has been suggested that fat from red meat is responsible, because high fat intake increases the concentration of cytotoxic lipids in the colon. Experimental studies have not unequivocally supported such a role for fat, however. Recently, we showed that dietary haem, which is abundant in red meat, increased colonic cytotoxicity and epithelial proliferation. In this study, we wanted to clarify whether dietary fat affects colon cancer risk by itself or by modulating the detrimental effects of haem on the colonic epithelium. Rats were fed control or haem-supplemented diets with 10%, 25% or 40% of the energy derived from fat for 14 days. Faeces were collected for biochemical analyses. Colonic cytotoxicity was determined from the degree of lysis of erythrocytes by faecal water. Colonic epithelial proliferation was measured *in vivo* using [^{3}H]thymidine incorporation. Increasing the fat content of the control diets stimulated faecal disposal of both fatty acids and bile acids. It also increased the concentration of fatty acids, but not that of bile acids, in faecal water in control rats. The cytolytic activity of faecal water and colonic epithelial proliferation were unaffected. Dietary haem increased faecal cation content and cytolytic activity of faecal water at all fat levels, suggesting that the colonic mucosa was exposed to high amounts of luminal irritants. This effect was smaller in rats on the low-fat diet. Dietary haem also increased colonic epithelial proliferation at all fat levels. The haem-induced effects were independent of fatty acids or bile acids in the faecal water. In western societies, 30–40% of ingested energy is supplied by dietary fat, so our results suggest that the association between consumption of red meat and risk of colon cancer is mainly due to its haem content, and is largely independent of dietary fat content.

Introduction

Colon cancer is the second most common cause of cancer deaths in western societies. It is an age-related disease, caused by a time-dependent accumulation of mutations in tumour suppressor genes and oncogenes, resulting in the subsequent transformation of normal epithelium into hyperproliferative tissue, adenoma and finally carcinoma (1). The predominant type of point mutation found in several genes of sporadic colon tumours is a G:C→A:T transition (2,3). These mutations can cause defective functioning of, for example, the APC (3) or p53 protein (4). C→T transitions are probably the result of endogenous processes and may not be caused by dietary mutagens (1). In this respect, the presence of irritants in the colonic lumen is important, because these compounds can damage the colonic mucosa and stimulate continuous regeneration of the epithelium (5), which increases the risk of endogenous mutation (1,6). Bile acids and fatty acids are considered to be the main irritants in the faecal stream and are thought to link the high-risk western style diet to colon cancer (7). The common hypothesis purports that the high saturated fat content of this diet increases the excretion of bile acids and fatty acids. However, several experimental studies have failed to show that dietary saturated fat enhances faecal bile acid excretion (8–10). Moreover, in a review of several large prospective cohort studies, colon cancer risk was associated with red meat consumption but not with total or animal fat intake (11). The authors of this review suggested that the risk-increasing effect of red meat could be due to its iron or its fat content. To our knowledge, the interaction between red meat and fat has never been investigated in an experimental study. Recently, we suggested that the association between red meat and colon cancer could be explained by the presence of haem in red meat (12). As an alternative to other hypotheses concentrating on heterocyclic amines and nitroso-compounds (see ref. 13 for review), we proposed that haem and its degradation products have cytotoxic effects in the colonic lumen and thus may induce compensatory hyperproliferation of the colonic epithelium. This hyperproliferation is commonly regarded as a risk factor for colon cancer (6). When haem was given as a dietary component to rats, we found a marked increase in the cytolytic activity of faecal water and in the proliferative activity of colonic epithelium (12). Haem is an amphiphilic molecule, and its cytotoxic effects may depend on other lipid-like components of the diet. Because there is doubt whether dietary fat increases faecal fatty acids or bile acids, we propose that fat may affect colonic epithelium by modulating the detrimental effects of haem. We therefore hypothesized that increasing the fat content of control diets (without haem) does not affect colonic epithelial proliferation, but that it enhances haem-induced faecal water cytolytic activity and colonic epithelial cell proliferation. To study this, rats were fed control or haem-supplemented diets with 10%, 25% or 40% of the energy derived from fat. The fatty acid composition of the blend of fat (82% palm oil and 18% corn oil) mimicked the ratio of saturated:mono unsaturated:polyunsaturated fatty acids (2:2:1) in a typical western human diet (14,15). Here we show that, irrespective of the dietary fat content, haem increased the cytolytic activity of faecal water and colonic epithelial proliferation, whereas fat itself had no effects. This suggests that haem, not fat, is primarily responsible for the association between consumption of red meat and colon cancer.

Table I. Composition of diets (g/kg)

Component	Low fat		Medium fat		High fat	
	Control	Haem	Control	Haem	Control	Haem
Casein	200	200	219	219	242	242
Dextrose	689	689	591	591	472	472
Fat	42	42	115	115	203	203
$CaHPO_4 \cdot 2H_2O$	3.44	3.44	3.76	3.76	4.16	4.16
Cellulose	20	20	21.9	21.9	24.2	24.2
Haem		0.82		0.90		0.99
Ferric citrate$\cdot 3H_2O$	0.38		0.42		0.45	
Mineral mix	35.0	35.0	38.3	38.3	42.3	42.3
Vitamin mix	10.0	10.0	10.9	10.9	12.1	12.1
Energy (MJ/kg diet)	16.02	16.02	17.53	17.53	19.37	19.37

The composition of the vitamin and mineral mixtures is according to the 1993 recommendations of the American Institute of Nutrition (16), except that calcium was omitted, tri-potassium citrate was added instead of KH_2PO_4, providing the same amount of potassium, and choline was added as choline chloride. The percentage of energy supplied by fat was 10%, 25% or 40% for the low-, medium- and high-fat diet, respectively.

Materials and methods

Animals and diets

The animal welfare committee of Wageningen University, Wageningen, The Netherlands approved the experimental protocol. Nine-week-old male, outbred Wistar rats (Harlan Horst/Wu, The Netherlands, specific pathogen free), mean body weight 280 g, were housed individually in metabolic cages in a room with controlled temperature (22–24°C), relative humidity (50–60%) and light–dark cycle (lights on from 6 a.m. to 6 p.m.). For 2 weeks, six groups of eight rats were fed purified diets, differing only in haem content and in amount of fat. The percentage of energy supplied by fat was 10%, 25% or 40% (the diets contained 42, 115 and 203 g fat/kg, respectively, which was exchanged for dextrose in the diet). Because the energy density of the diets increased with their fat content, the amount of all ingredients (except fat and dextrose) added to the diets was adjusted to provide equal nutrient density (g/kJ) in all diets. The composition of the diets is given in Table I. Low-, medium- and high-fat diets were supplemented with 1.26, 1.39 and 1.52 mmol haem (haemin; Sigma-Aldrich Chemie, St Louis, MO, USA) or ferric citrate (controls) (BDH, Brunschwig Chemie, Amsterdam, The Netherlands) per kilogram of diet, respectively. Calcium was added as $CaHPO_4 \cdot 2H_2O$ (Fluka Chemie, Buchs, Switzerland). Other minerals, vitamins and choline (as choline chloride) (adjusted for the energy density of the diets) were added to the diets according to the 1993 recommendations of the American Institute of Nutrition (16). Food and demineralized drinking water were supplied *ad libitum*. Food intake and body weights were recorded every 2–4 days. Faeces were collected quantitatively during days 11–14 of the experiment and were frozen at –20°C.

In vivo colonic epithelial cell proliferation

After the rats had been fed the experimental diets for 14 days, the DNA and protein content of the colonic scrapings and the proliferative activity of the whole colonic epithelium, quantified by the amount of [methyl-^{3}H]thymidine (Amersham International, Amersham, UK) incorporated per microgram of DNA, were determined as described before (12).

Preparation of faecal water

Faeces were freeze-dried after collection. Faecal water was prepared by reconstituting the homogenized freeze-dried faeces with appropriate amounts of double-distilled water to provide samples with an osmolarity of 300 mosmol/l, as described previously (12). Faecal water samples were stored at –20°C until analysis.

Cytolytic activity of faecal water

The cytolytic activity of faecal water was quantified by potassium release from erythrocytes, as described by Govers *et al.* (17), with some modifications. For this purpose, 10 or 20 µl of faecal water was mixed with saline to a volume of 80 µl. After incubation for 5 min at 37°C, 20 µl of a washed human erythrocyte suspension was added (final haematocrit 5%) and incubated for 15 min at 37°C. Cytolytic activity was measured as described before (17). The relevance of this bioassay with erythrocytes for effects on intestinal epithelial cells was shown by the high correlation coefficient ($r = 0.97$) between the lytic effects of mixtures of bile acids and fatty acids on human erythrocytes and on the human colon carcinoma-derived Caco-2 cells (18).

Analysis of faecal water

To determine free fatty acids and bile acids in faecal water, acidified faecal water (final HCl concentration 1 M) was extracted three times with 5 vols diethyl ether. The diethyl ether phase was dried under nitrogen and the extract was resolubilized in ethanol. Free fatty acids were determined using a colorimetric enzymatic assay (NEFA-C kit; Wako Chemicals, Neuss, Germany) and bile acids were measured with a fluorescent enzymatic assay, as described earlier (19). The amount of haem in the faecal water was measured with a modified HemoQuant assay (20) using haemin as a standard. Mean ± SEM recovery of haem and protoporphyrin was 95% ± 6% and 83% ± 3%, respectively.

Analysis of total faeces

The amounts of sodium and potassium in faeces were determined using atomic emission spectrophotometry as described previously (12). Faecal ammonia was measured using a urea nitrogen kit (Sigma Diagnostics, St Louis, MO, USA), omitting the incubation step with urease (12). The percentage water of faeces was calculated assuming that the total amount of sodium, potassium, ammonia and their negatively charged counterions provided an osmolarity of 300 mosmol/l in faeces (21). To determine haem in whole faeces, an acidified chloroform–methanol extract (22) (final HCl concentration 1 M) was obtained from ~20 mg of faeces. The chloroform phase of the samples was dried under nitrogen and resolubilized in 0.45 ml 250 mM KOH. Subsequently, 0.45 ml double-distilled water, 3.75 ml 2-propanol and 0.75 ml 1.15 M HCl were added. The samples were centrifuged for 10 min at 1500×*g* and the supernatants were assayed for their haem content (20). Using this procedure, mean ± SEM recovery of haem and protoporphyrin was 109% ± 7% and 95% ± 5%, respectively. Free fatty acids and bile acids in faeces were determined as described previously (5).

Statistics

Results are presented as means ± SEM ($n = 8$). A commercially available package (Statistica 5.5 StatSoft Inc., Tulsa, OK, USA) was used for all statistics. To evaluate independent effects of increasing doses of dietary fat, one-way analysis for control groups only was performed. To test whether dietary fat interacted with effects of haem, two-way analysis of variance was performed with the fat content and haem content of the diets as independent variables. In case of treatment effects, each haem group was compared with its fat-matched control. Distribution of data was evaluated using normal probability plots. When data were distributed normally, Student's *t*-test with Bonferroni correction was used to test for differences between means (two-sided). When data were not normally distributed, differences between the haem groups and their fat-matched control groups were tested using the non-parametric Mann–Whitney *U*-test. Bonferroni correction was made for the number of equations ($n = 3$). Differences were considered statistically significant when P was <0.05 (two-sided).

Table II. Daily intake of food, energy, and haem and growth of the animals

Fat content of diet	Group	Food intake (g/day)	Energy intake (kJ/day)	Haem intake (μmol/day)	Growth (g/day)
Low	control	21.7 ± 0.2	348 ± 4	0	3.9 ± 0.2
	haem	20.8 ± 0.4	333 ± 6	26.2 ± 0.5	3.1 ± 0.4
Medium	control	18.9 ± 0.4	331 ± 8	0	3.5 ± 0.3
	haem	19.0 ± 0.3	334 ± 6	25.9 ± 0.5	3.4 ± 0.3
High	control	17.1 ± 0.3	331 ± 6	0	3.4 ± 0.3
	haem	17.1 ± 0.5	331 ± 9	26.0 ± 0.7	3.2 ± 0.2

The percentage of energy supplied by fat was 10%, 25% or 40% for the low-, medium- and high-fat diet, respectively. Food intake and growth of the animals were recorded every 2–3 days. Values represent means ± SEM (n = 8).

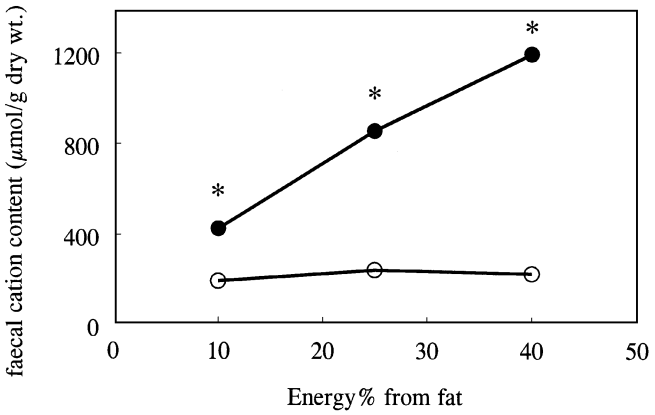

Fig. 1. Effect of haem and increasing concentrations of dietary fat on the concentration of cations (Na^+, K^+ and NH_4^+) in the faeces. Results are given as means (n = 8). The standard errors were smaller than the size of the symbols. ○, of control (non-haem) rats; ●, of haem-fed rats. Two-way analysis revealed a strong interaction between haem and the fat content of the diet on faecal cation content ($P < 0.001$). *Significant difference between haem-fed rats and their fat-matched controls.

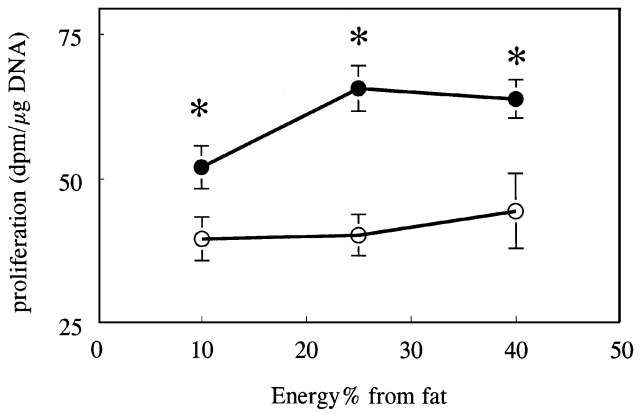

Fig. 2. Effect of haem and increasing concentrations of dietary fat on colonic epithelial proliferation, determined by incorporation of [methyl-^{3}H]thymidine in the mucosa. Results are given as mean ± SEM (n = 8). ○, Mucosa of control (non-haem) rats; ●, mucosa of haem-fed rats. *Significant difference between haem-fed rats and their fat-matched controls.

Results

Animals and diets

Table II shows the effects of the different diets on daily intake of food, energy and haem and on growth of the animals. Daily food intake was lower when the amount of fat of the diet was increased ($P < 0.001$); this was independent of the presence of haem. However, the energy density of the diet increased with their fat content, so daily energy intake was equal among all groups. This was also the case for haem: all three haem-fed groups consumed the same amount of haem per day. Neither the haem content nor the fat content of the diet affected growth of the animals.

Effects of diets on faecal cation content

A striking observation was that the faeces of rats fed the medium- and high-fat haem diets, but not the low-fat haem diet, were softened, whereas faeces of rats in all three non-haem groups had a normal appearance. Because this might indicate that the composition of the diets affected colonic absorption of cations and water, we quantified the hydration of faeces by measuring the faecal concentration of cations (Na^+, K^+ and NH_4^+) (Figure 1). Fat itself had no effect on concentration of cations in the faeces. In contrast, dietary haem increased the faecal cation content at all three fat levels, and there was a strong interaction with the dietary fat content ($P < 0.001$ for the interaction). Consequently, the calculated percentage wet weight of the faeces was equal among all three non-haem groups (55% ± 3%, 60% ± 2% and 59% ± 2% for the low-, medium- and high-fat diet, respectively), whereas it was greatly increased by the addition of haem to the diets: 73% ± 3%, 85% ± 0% and 89% ± 0%, respectively.

Effect of dietary haem and fat on colonic epithelial proliferation

Figure 2 shows the effect of the dietary treatments on the proliferation of colonic epithelium. Epithelial proliferation was not increased when the dietary fat content of the control diets was raised from 10% energy to 40% energy. Two-way analysis of variance showed that there was a haem-induced increase in colonic epithelial proliferation, independent of dietary fat content ($P < 0.001$ for the haem effect). The effect of haem tended to be lower in rats on the low-fat diet. Neither haem nor fat affected the DNA or protein content or the DNA:protein ratio of the colonic scrapings compared with control values (DNA: 1029 ± 80 μg/scraping, protein: 13.0 ± 0.7 mg/scraping, DNA:protein ratio: 81 ± 7 μg/mg).

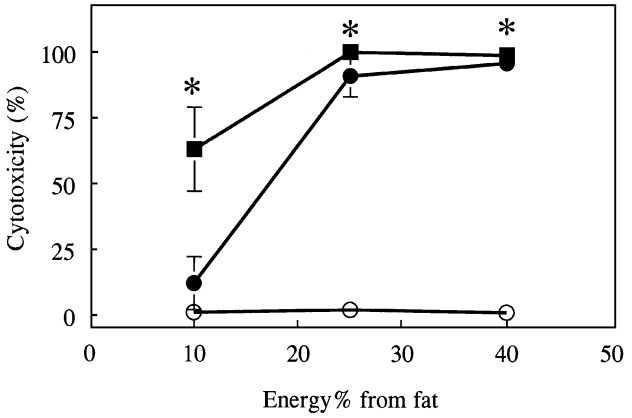

Fig. 3. Effect of haem and increasing concentrations of dietary fat on the cytolytic activity of faecal water. Values represents means ± SEM (n = 8). ○, 10 µl of faecal water from control (non-haem) rats (cytolytic activity of 20 µl: results with control faecal water are not shown, because they were not different from those with 10 µl); ●, 10 µl of faecal water from haem-fed rats; ■, 20 µl of faecal water from haem-fed rats. Two-way analysis revealed a strong interaction between haem and the fat content of the diet on the cytolytic activity of faecal water ($P < 0.001$). *Significant difference between haem-fed rats and their fat-matched controls.

Cytolytic activity of faecal water

Figure 3 shows that the cytolytic activity of faecal water from the control groups was not sensitive to variations in the fat content of the diet. In contrast, dietary fat did affect the haem-induced cytolytic activity of faecal water. When 10 µl was used in the assay, the haem-induced increase in cytolytic activity was very large on the medium- and high-fat diets, whereas this effect was much smaller in rats on the low-fat diet ($P < 0.001$ for the interaction). When 20 µl was used in the assay instead of 10 µl, the cytolytic activity of faecal water from the low-fat haem group was also strongly stimulated, whereas faecal water from the control groups still showed no cytolytic activity ($P = 0.01$ for the interaction). Thus, also in rats on a low-fat diet, haem considerably increased the cytolytic activity of the faecal water.

Effect of diets on the composition of faecal water

To determine the factors responsible for the diet-induced changes in faecal cytolytic activity, we analysed the composition of faecal water (Table III). Control groups were considered first; it appeared that the pH of the faecal water was not significantly affected by increasing the fat content of the diet. Dietary fat increased the concentration of fatty acids ($P < 0.001$), but not of bile acids, in faecal water of controls. The haem content of control faecal water samples was very low, with no differences between the dietary fat levels. When haem was added to the diets, the pH decreased at all fat levels ($P = 0.002$), but this did not depend on the fat content of the diet. Although the concentration of fatty acids was increased by dietary haem in rats on the low-fat diet, there was no significant overall haem-induced increase in fatty acid concentration. The bile acid concentration in faecal water was reduced by dietary haem in rats on the medium- and high-fat diets, but not in those on the low-fat diet ($P = 0.008$ for the interaction). Addition of haem to the diet increased the haem content of faecal water drastically ($P < 0.001$), without significant differences between the fat levels.

Composition of total faeces

Dietary fat did not induce a significant increase in daily faecal dry weight in rats fed the control diets (Table IV). In these groups, increasing the fat content of the diets significantly increased daily faecal output of both fatty acids ($P < 0.001$) and bile acids ($P = 0.001$). Haem output was very low in these groups, and did not depend on the fat content of the diet. Adding haem to the diets increased the dry weight of faeces excreted each day ($P = 0.02$), independent of dietary fat content. It also stimulated daily faecal output of fatty acids ($P = 0.008$) and of bile acids ($P = 0.03$) in the faeces, and for both components there was no significant interaction with fat. Faecal output of haem was slightly higher in rats on the low-fat diet than in those on the medium- and high-fat diets, but this did not reach statistical significance. Metabolism of haem, calculated as the amount of ingested haem minus faecal output of haem, was 10.8 ± 1.1, 13.1 ± 0.7 and 13.6 ± 1.0 µmol/day for the low-, medium- and high-fat haem diets, respectively. These differences were not significantly different from each other.

Discussion

As mentioned in the Introduction, there were two main questions to be answered in this study. Firstly, does feeding diets with increasing fat content affect the proliferative activity of rat colonic epithelium? Secondly, does fat modulate the deleterious effects of dietary haem? To answer the first question, only control diets without haem were considered. The results of this study do not support the hypothesis that dietary fat by itself is involved in the pathogenesis of diet-induced colorectal cancer. The generally accepted hypothesis states that higher intake of fat increases the excretion of fatty acids and bile acids into the colonic lumen, where they can be cytotoxic to the colonic epithelium (7). In accordance with this proposed mechanism, increasing the fat content of control diets in the present study did indeed enhance the faecal excretion of these lipids. However, as shown by Lapré *et al*. (23), only water-soluble surfactants, and not their concentrations in whole faeces, determine the cytolytic activity of faecal water. Enhancing the fat content of control diets increased the concentration of fatty acids, but not of bile acids, in faecal water. Apparently this was insufficient to affect the colonic mucosa, because faecal water from all non-haem groups had very low cytolytic activity. This indicates that, in the present study, dietary fat did not change the potency of faecal water to damage colonic epithelium. This is supported by the observation that increasing the fat content of control diets did not affect faecal cation content. High faecal cation content could be the result of luminally induced damage to the colonic mucosa (24–26). In the present study, in accordance with the lack of effect of faecal water on cytolytic activity, proliferation of the colonic mucosa was not affected. Thus, although faecal excretion of bile acids and fatty acids was increased by dietary fat, their effect on colonic epithelium was minimal.

There is no consistent evidence for the hypothesis that dietary fat increases colon cancer risk by enhancing the excretion of cytotoxic lipids into the colonic lumen. In

Table III. pH, bile acids, fatty acids and haem content in faecal water

Fat content of diet	Group	pH	Fatty acids[a] (mM)	Bile acids[b] (mM)	Haem[c] (mM)
Low	control	7.62 ± 0.06	1.21 ± 0.13	1.97 ± 0.19	11 ± 1
	haem	7.35 ± 0.13	2.27 ± 0.29*	1.68 ± 0.13	336 ± 31*
Medium	control	7.50 ± 0.08	1.60 ± 0.26	2.16 ± 0.21	10 ± 1
	haem	6.93 ± 0.11*	1.46 ± 0.10	1.17 ± 0.11*	264 ± 20*
High	control	7.35 ± 0.13	3.15 ± 0.61	2.21 ± 0.12	10 ± 2
	haem	7.19 ± 0.19	3.01 ± 0.49	0.98 ± 0.08*	235 ± 43*

The percentage of energy supplied by fat was 10%, 25% or 40% for the low-, medium- and high-fat diet, respectively. Values represent means ± SEM (n = 8).
[a]Significant fat-induced increase in control (non-haem) diets.
[b]Statistically significant interaction between haem and the fat content of the diet ($P < 0.05$).
[c]One-way ANOVA revealed no significant differences in haem content of faecal water from rats on the low-, medium- and high-fat haem groups.
*Statistically significant difference between haem-fed rats and their fat-matched non-haem controls.

Table IV. Effect of diets on daily faecal output of dry weight, haem, fatty acids, and bile acids

Fat content of diet	Group	Dry weight[a] (g dry wt./day)	Fatty acids[a,b] (μmol/day)	Bile acids[a,b] (μmol/day)	Haem[c] (μmol/day)
Low	control	0.60 ± 0.02	90 ± 12	10.7 ± 0.6	0.1 ± 0.1
	haem	0.64 ± 0.04	143 ± 19	15.1 ± 1.3*	15.3 ± 1.2*
Medium	control	0.66 ± 0.03	155 ± 13	13.1 ± 1.0	0.1 ± 0.0
	haem	0.73 ± 0.05	181 ± 21	15.3 ± 1.0	12.8 ± 1.0*
High	control	0.66 ± 0.02	175 ± 15	17.0 ± 1.3	0.4 ± 0.3
	haem	0.78 ± 0.05	231 ± 32	16.5 ± 1.3	12.4 ± 1.2*

The percentage of energy supplied by fat was 10%, 25% or 40% for the low-, medium- and high-fat diet, respectively. Values represent means ± SEM (n = 8).
[a]Two-way ANOVA showed fat-independent effect of dietary haem on dry weight of faeces produced (P = 0.02), on faecal excretion of fatty acids (P = 0.008) and bile acids (P = 0.03).
[b]Significant increase in fatty acids ($P < 0.001$) and in bile acids (P = 0.001) due to dietary fat in control (non-haem) groups.
[c]One-way ANOVA for the haem groups revealed no significant differences in faecal haem output between low-, medium- and high-fat haem groups.
*Statistically significant difference between haem-fed rats and their fat-matched controls.

animal studies, colonic epithelial proliferation was not affected (27) or was even decreased (28) by high-fat diets. Faecal bile acid excretion was stimulated by high-fat diets in some (29,30), but not all (31) studies. Human experimental data with regard to the fat–bile acid hypothesis are limited. When studying the effects of different amounts of dietary fat on faecal bile acids, no convincing evidence was obtained for a stimulatory effect of fat on faecal bile acid excretion (8–10). Only one study has assessed the effect of increasing doses of dietary fat on colonic epithelial proliferation in humans (32). In that study, dietary fat increased proliferation of the colonic epithelium only when it was given as a single fat bolus 6 h after the last meal, but not when the same amount of fat was consumed during meals.

In contrast to the lack of effect of dietary fat, haem increased the proliferative activity of the colonic epithelium. Like fat, haem increased the faecal excretion of fatty acids and also of bile acids in rats on the low-fat diet. The reason for this is not known, however, and the results indicate that the haem-induced increase in these cytotoxic lipids is not relevant to the deleterious effects of haem. The present study confirms the proposed mechanism through which haem exerts its effects on colonic epithelium (12). Consumption of haem-containing diets strongly increased the cytolytic activity of faecal water. Together with the increase in faecal cation content, this suggests that the colonic mucosa was damaged when rats were fed haem-supplemented diets. We have shown earlier (5) that the cytotoxicity of faecal water and epithelial damage were highly correlated with colonic epithelial proliferation, suggesting that loss of cells is compensated for by an increased proliferative activity of the colonic epithelium. Indeed, consumption of haem-containing diets by rats increased the proliferation of colonic epithelium.

Though dietary haem strongly increased the cytolytic activity of faecal water, it did not increase the concentration of fatty acids in faecal water. In addition, the concentration of bile acid in the faecal water of haem-fed rats was even lower than that in controls, probably due to dilution by the increased amount of water in the faeces. We also measured the pH of the faecal water, because the red blood cells in the bioassay are sensitive to variations in the pH of the incubation medium. However, simple acid-induced lysis can be excluded, because the pH of faecal water from the haem-supplemented groups was within the pH range of controls. These results indicate that a hitherto unknown, highly cytolytic factor was formed in the intestine of haem-fed rats, which could be responsible for the mitogenic effect of dietary haem. We have shown before that the cytolytic activity of faecal water and proliferation of colonic epithelium only increased when haem was present in the diet, and not when haem was replaced by equimolar amounts of its

separate constituents, inorganic iron or protoporphyrin (12). This indicates that the presence of iron is a prerequisite for the formation of the cytolytic factor, but only when it is in the form of haem. This is in contrast to the findings of Lund *et al*. (33), who reported a small increase in the number of mitotic cells in rat colonic crypts when the iron content of the diet was increased 3.5 times using ferric sulfate.

The second main question of this study was whether the effects of haem were dependent on the fat content of the diet. Haem-induced cytolytic activity was lower in rats on a low-fat diet, suggesting that in these rats, exposure of the colonic mucosa to luminal irritants was lower. In addition, the haem-induced increase in faecal cation content, reflecting epithelial damage, was strongly fat-dependent. The ultimate physiological effect through which haem is supposed to increase colonic cancer risk is by increasing damage to epithelial cells. In accordance with this, we found that dietary haem at all fat levels significantly increased the proliferative activity of the colonic epithelium, although haem had slightly less effect in rats on the low-fat diet. It should be noted that the concentrations of dietary haem used here were relatively high compared with concentrations that one would expect to find in humans (34). If lower concentrations of dietary haem also have effects like those described here, we cannot exclude the possibility that these effects could be affected by dietary fat content. A recent clinical trial considered the effect of a reduction in dietary fat, in combination with an increase in fibre, on the recurrence of colorectal adenomas. Against an almost stable background of red meat intake, and thus haem intake, no protective effect was found when the contribution of fat to total energy intake was decreased from 36% to 24% (35).

In summary, this study showed that increasing dietary fat content from 10% energy to 40% energy in the absence of haem does not affect the cytolytic activity of colonic contents or epithelial proliferation, whereas both parameters are increased by dietary haem. The effect of haem on proliferation was slightly lower in rats on the low-fat diet, corresponding to a smaller haem-induced increase in cytolytic activity of faecal water. Considering that, in western societies, 30–40% of ingested energy is typically supplied by dietary fat (14,15), our results suggest that the epidemiological association between consumption of red meat and risk of colon cancer is mainly due to haem, and is unlikely to be dependent on the fat content of the diet.

Acknowledgements

The authors wish to thank Bert Weijers and Maria Faassen-Peters (Small Animal Research Centre, Wageningen University, The Netherlands) for their skilful biotechnical assistance. This study was supported by grant 904-62-167 from The Netherlands Organization for Scientific Research, Medical Sciences.

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Received May 5, 2000; revised July 20, 2000; accepted July 31, 2000