

# CHAPTER 2

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## **Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme**

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## ABSTRACT

Intake of a Western diet with a high amount of red meat is associated with a high risk for colon cancer. We hypothesize that heme, the iron-carrier of red meat, is involved in diet-induced colonic epithelial damage, resulting in increased epithelial proliferation. Rats were fed purified control diets, or purified diets supplemented with 1.3  $\mu\text{mol/g}$  of heme (ferriheme), protoporphyrin IX, ferric citrate or bilirubin ( $n=8/\text{group}$ ) for 14 days. Feces were collected for biochemical analyses. Fecal cytotoxicity was determined from the degree of lysis of erythrocytes by fecal water. Colonic epithelial proliferation was measured in vivo using [ $^3\text{H}$ ]-thymidine incorporation into colonic mucosa.

Colonic epithelial proliferation in heme-fed rats was significantly increased compared to control rats ( $55.2 \pm 5.8$  vs.  $32.6 \pm 6.3$  dpm/ $\mu\text{g}$  DNA, (mean  $\pm$  SE),  $p<0.05$ ). Fecal water of the heme-group was highly cytotoxic compared to controls ( $100 \pm 0\%$  vs.  $2 \pm 1\%$ ,  $p<0.001$ ), though the concentrations of cytotoxic bile acids and fatty acids were significantly lower. Organic iron was significantly increased compared to controls ( $257 \pm 26$  vs.  $80 \pm 21$   $\mu\text{M}$ ,  $p<0.001$ ). Spectrophotometric analyses suggest that this organic iron is heme-associated. Thiobarbituric acid reactive substances (TBARS) were greatly increased in fecal water of heme-fed rats compared to controls ( $177 \pm 12$  vs.  $59 \pm 7$   $\mu\text{M}$ ,  $p<0.05$ ). Heme itself could not account for the increased cytotoxicity, as addition of heme to fecal water of the control group, equimolar to the organic iron content of fecal water of the heme group, did not influence cytotoxicity. Hence, an additional, heme-induced cytotoxic factor is involved, which may be modulated by the generation of luminal reactive oxygen species. Protoporphyrin IX, ferric citrate, and bilirubin did not increase proliferation and cytotoxicity. In conclusion, dietary heme leads to formation of an unknown, highly cytotoxic factor in the colonic lumen. This suggests that, in heme-fed rats, colonic mucosa is damaged by the intestinal contents. This results in a compensatory hyperproliferation of the epithelium, which supposedly increases the risk for colon cancer.

## INTRODUCTION

In Western societies, colon cancer is one of the major causes of cancer death. 110,000 new cases are diagnosed every year in the United States, and 55,000 people die annually because of this disease. There is a wide geographic variation in incidence, with a 20-fold variance worldwide (1). Though it is recognized that genetic factors are important determinants for the genesis of colorectal cancer in individuals (2), it appears that differences in colon cancer incidence are mainly attributable to environmental factors (3). Epidemiological studies have shown that especially people with a Western-style diet (high meat, high fat, low fiber) are at high risk for colon cancer. Incidence increases in countries with a high meat consumption (4). More specifically, red meat, but not white meat, increases risk for colon cancer (5,6).

To explain this latter association, several hypotheses have been proposed, which were summarized recently (7). First, risk for colon cancer is epidemiologically linked to the consumption of well done fried meat, probably due to the presence of heterocyclic aromatic amines. These compounds are true carcinogens in animal models. However, the contribution of heterocyclic amines to human colon cancer incidence is thought to be very low, as doses required for carcinogenicity in animal studies exceed the daily human intake by several orders of magnitude. Furthermore, the hypothesis is challenged by the fact that levels of heterocyclic amines in cooked white meat exceed levels in red meat (8). Therefore, heterocyclic amines cannot explain the differential effects of red and white meat. Secondly, in the gastrointestinal tract, the reaction of nitrosating agents, like NO and N<sub>2</sub>O<sub>3</sub>, with amines can form N-nitroso compounds. Though proven to be mutagenic in vitro, carcinogenicity of these compounds in humans is still under debate. According to the third hypothesis, consumption of meat increases the intake of fat, which itself is often regarded as a risk factor for colon cancer. Dietary fat is thought to act, at least in part, by increasing the intracolonic concentrations of membrane-damaging bile acids and fatty acids or via the production of the potentially mitogenic diacylglycerol. Large prospective cohort studies, however, have shown that the association of colon cancer and red meat cannot be explained solely by the fat content of the meat (9). The fourth hypothesis suggests a role for dietary iron in colorectal carcinogenesis, because of its catalytic activity in formation of oxygen radicals (10,11). However, in animal studies, in which different forms of iron were tested, a clear role for iron in colon cancer could not be established (12,13,14).

It should be noted that, although iron from meat is mainly in the form of heme (content in beef is approximately 1.5 μmol heme/g dry weight, (15)), specific effects of the heme molecule on the colonic epithelium have not hitherto been considered. Heme absorption is very low and most ingested heme is therefore delivered to the colon. It was shown that after consumption of red meat, but not of chicken or fish (which have a low heme content), heme could be recovered from the feces (15). Heme is an amphipathic molecule with a bulky, hydrophobic tetrapyrrole ring structure with two propionic acid side chains. Earlier studies in our laboratory have shown that other amphipathic molecules, like bile acids and fatty acids, can cause epithelial damage, resulting in a compensatory epithelial hyperproliferation (16). Furthermore, it was shown that heme is cytotoxic towards mammalian cells in vitro (17). Therefore, we hypothesized that dietary heme or its metabolic degradation products causes cytotoxic effects in the colonic lumen, which may affect the proliferation of the colonic epithelium. We tested this in rats and compared heme with equimolar amounts of other tetrapyrroles, protoporphyrin IX and bilirubin, which are normal physiological heme metabolites (15, 18). Furthermore, as heme is an important iron-carrier, ferric citrate was also included in the study.

## MATERIALS AND METHODS

### **Animals and diets.**

The experimental protocol was approved by the animal welfare officer of the Agricultural University, Wageningen, The Netherlands. Nine-week-old male, outbred Wistar rats (Harlan Horst/Wu, specific pathogen free), mean body weight  $325 \pm 2$  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 AM to 6 PM). During two weeks, 5 groups of 8 rats were fed purified diets. The control diet contained per kg, 200 g casein, 617 g dextrose, 115 g fat (82% palm oil and 18% corn oil), 20 g cellulose and 20 mmol calcium phosphate ( $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , Fluka Chemie, Buchs, Switzerland). The fatty acid composition of the blend of palm oil and corn oil mimics the ratio of saturated to monounsaturated to polyunsaturated fatty acids (44:38:18) in a human Western diet. Other minerals and vitamins, including choline, were added to the diets according to the recommendations of the American Institute of Nutrition 1993 (19). For the experimental groups, diets were supplemented with  $1.3 \mu\text{mol/g}$  of heme (hemin was used for this purpose), protoporphyrin IX, bilirubin (all Sigma-Aldrich Chemie, St. Louis, MI, US) or ferric citrate (BDH, Brunschwig Chemie, Amsterdam, The Netherlands). Food and demineralized drinking water were supplied ad libitum. Food intake and body weights were recorded every 2-4 days. Feces were collected quantitatively during days 11-14 of the experiment and were frozen immediately at  $-20^\circ\text{C}$ .

### ***In vivo* colonic proliferation.**

After the experimental feeding period of 14 days, non-fasted rats were injected i.p. with [methyl- $^3\text{H}$ ]thymidine (specific activity 25 Ci/mmol; dose  $100 \mu\text{Ci/kg}$  body weight; Amersham International, Amersham, UK) in 154 mM NaCl. After 2 h, they were killed by  $\text{CO}_2$  inhalation, and the colon was removed and longitudinally opened. Colonic contents were removed by rinsing with 154 mM KCl and the mucosa was scraped off with a spatula and homogenized in 1 mL 154 mM KCl (Ultraturrax Pro200: Pro Scientific Inc., Monroe, CT, US). The scrapings were analyzed as described previously (20). For protein determinations,  $100 \mu\text{L}$  of homogenate was diluted ten-fold with double-distilled water and deoxycholic acid was added (final concentration 0.15 mg/mL). After incubation for 10 min at room temperature, protein was precipitated with trichloroacetic acid (final concentration 60 mg/mL). Samples were centrifuged for 15 min at 3,000 g and the pellet was resolubilized in sodium dodecyl sulfate (50 mg/mL) in 100 mM NaOH. Protein was quantified, according to Smith et al. (21), using the BCA Protein Assay Kit (Pierce, Rockford, IL, US) with bovine serum albumin as standard.

### **Fecal water preparation.**

Fecal water was prepared by reconstituting a small amount of freeze-dried feces with double-distilled water to 30% dry weight. After homogenizing, samples were incubated for 1 h in a shaking waterbath at  $37^\circ\text{C}$ , with mixing every 15 min, followed by centrifugation for 10 min at 15,000 g. Then, supernatants were centrifuged at 15,000 g for another 2 min. The

supernatant was aspirated and osmolarity was measured (Osmomat 030-D, Gonotec, Berlin, Germany). If osmolarity differed from 300 mosmol/L, another portion of freeze dried feces was reconstituted with double-distilled water, adjusting the percentage dry weight to obtain fecal water with an osmolarity of 300 mosmol/L. Fecal water was stored at -20°C until analysis.

### **Cytotoxic activity assay.**

Cytotoxic activity of fecal water was quantified by potassium-release from erythrocytes, as described by Govers et al.(22), with the following modifications. The dose-dependent cytotoxicity of pooled fecal water of control and of heme groups was determined by supplementing increasing volumes of fecal water with saline to a total volume of 80  $\mu$ L. After preincubation for 5 min at 37 °C, 20  $\mu$ L of a washed human erythrocyte suspension were added (final hematocrit 5%) and incubated for 15 min at 37 °C. Cytotoxicity was measured as described before (22). Subsequently, differences between experimental groups were quantified by testing cytotoxicity of 10  $\mu$ L of fecal water. Finally, to test whether fecal cytotoxicity of heme-fed rats was dependent on oxygen, cytotoxicity of increasing volumes of fecal water of the heme group was determined, as described above, in an anaerobic cabinet (Coy Laboratory products Inc., Ann Arbor, MI, US). Simultaneously, equal amounts were tested at ambient atmosphere.

### **Total feces analyses.**

For total iron determination, feces were dry-ashed for 8 h at 550°C (Heraeus, Eurotherm 815, Boom Meppel, The Netherlands), followed by destruction (20 min, 180 °C) with 500  $\mu$ L perchloric acid (70%) and 100  $\mu$ L H<sub>2</sub>O<sub>2</sub> (30%). After dilution in double-distilled water, iron was measured using an atomic absorption spectrometer (Perkin Elmer Corp., model 1100, Norwalk, CT). Recovery of added ferric citrate was 95%  $\pm$  7% and that of hemin was 85%  $\pm$  5%.

To measure sodium and potassium, feces were treated with 5% trichloroacetic acid for 1 h at room temperature and centrifuged for 2 min at 14,000 g. The supernatants were diluted with 0.05% CsCl, and sodium and potassium were measured by atomic emission spectrometry. For determination of the ammonia content, feces were incubated for 15 min at 37 °C in 5% perchloric acid. Samples were centrifuged for 2 min at 14,000 g and ammonia was measured in the supernatant using the Sigma Urea Nitrogen Kit (Sigma Diagnostics, No. 640, St. Louis, MO, US), omitting the incubation step with urease. The percentage water of feces was calculated assuming that the total amount of sodium, potassium, ammonia and their negatively charged counterions provided an osmolarity of 300 mosmol/L in feces (23).

### **Analyses of fecal water.**

To determine total iron in fecal water, samples were treated as described for feces, except that the dry-ashing step was omitted and 50  $\mu$ L perchloric acid and 20  $\mu$ L H<sub>2</sub>O<sub>2</sub> were used for destruction. For inorganic iron determination, fecal water was incubated in 10%

trichloroacetic acid for 20 min at 90 °C and centrifuged at 14,000 g for 2 min. The supernatant was diluted in double-distilled water and inorganic iron was measured by atomic absorption spectrometry. Organic iron was calculated as the difference between total iron and inorganic iron. Using these procedures, recovery of added ferric citrate as inorganic iron was  $97\% \pm 2\%$  and recovery of hemin as organic iron was  $92\% \pm 7\%$ . For determination of free fatty acids and bile acids in fecal water, acidified fecal water (final HCl concentration 1M) was extracted three times with 5 volumes of 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 (20). For spectrophotometric analyses, an acidified chloroform-methanol extract (final HCl concentration 1M) was obtained (24). The chloroform phase was dried under nitrogen and resolubilized in methanol. An absorption spectrum was recorded from 300 to 700 nm on a spectrophotometer (Perkin Elmer, Lambda 2, Norwalk, MO, US).

To evaluate lipid peroxidative processes in the lumen, thiobarbituric acid-reactive substances (TBARS) in fecal water were measured according to Ohkawa et al. (25). Briefly, fecal water was diluted ten-fold with double-distilled water and 100  $\mu$ l of this diluted fecal water was mixed with 100  $\mu$ L 8.1% sodium dodecyl sulfate and 1000 $\mu$ L 0.5% TBA in 10% acetic acid (pH=3.5). For blanks, TBA was omitted from the assay. After heating for 60 min at 95 °C, TBARS were extracted with 1.2 ml n-butanol. The absorbance of this butanol extract was measured at 532 nm. The amount of TBARS was calculated as malondialdehyde (MDA) equivalents using 1,1,3,3,-tetramethoxypropane as standard.

### **Statistics.**

Results are presented as means  $\pm$  SEM (n=8). A commercially available package (SPSS/PC + v2.0, SPSS Inc., Chicago, IL.) was used for all statistics. In the case of normally distributed data, one-way analysis of variance was performed, followed by Fisher's least significant difference test (LSD, two-sided) to test for significant differences between means of dietary treatments and control diet. When data of groups showed unequal variances, distribution of data was normalized using ln-transformation and data were treated as described above. If variances were still unequal, the nonparametric Kruskal-Wallis test was used and differences between means were tested with the Mann-Whitney U test for significance (two-sided). For all parameters, data from each experimental group were compared with control data only.

## **RESULTS**

No significant differences were observed between groups in food intake (mean 19.9 g/day) and initial (mean 323 g) and final (mean 376 g) body weight during the experimental period. The effect of the different dietary treatments on fecal parameters is shown in Table 1. It

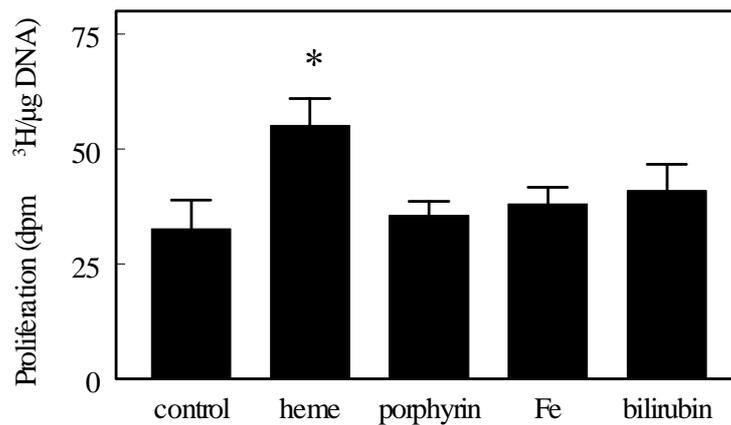
shows that in both high-iron groups, fecal iron was significantly increased, whereas it was not affected by protoporphyrin IX or bilirubin. From these data it can be calculated that apparent iron absorption from the control diet was approximately 12%. Iron supplementation by either heme or ferric citrate did not increase the apparent iron absorption. Thus, a large majority of dietary heme iron and ferric citrate reached the colon. There, only heme iron had striking effects on fecal parameters, not shown by the other diets (Table 1). Daily fecal output (total dry weight) was higher when heme was added to the diet. During the experiment, softening of feces of heme-fed rats was observed, while feces from other experimental groups had appearance, similar to control feces. These symptoms may reflect a disturbance of the absorption or secretion function of the colon. Therefore, we measured major fecal cations (sodium, potassium, and ammonia), and found that these were greatly increased by dietary heme. Consequently, net absorption of water was lower, as reflected by a higher fecal wet weight (%) in the heme-group.

Because the colonic epithelium is sensitive to diet-induced changes in the lumen (20,26), we next determined the proliferation of the colonic epithelial cells. Figure 1 shows that the heme diet significantly increased proliferation compared with the control diet, while proliferation was not affected by the other dietary treatments. DNA and protein content did not differ among the groups (DNA  $874 \pm 83$   $\mu$ g/scraping, protein  $9.1 \pm 1.0$  mg/scraping).

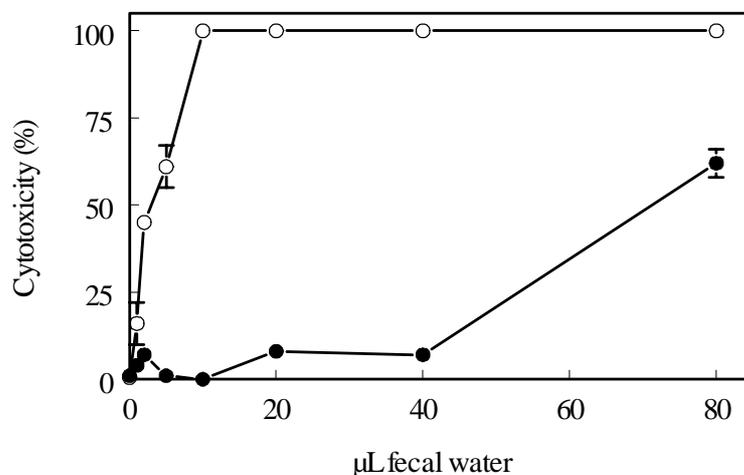
**Table 1.** Effects of dietary tetrapyrroles on fecal parameters<sup>a</sup>.

Diet	total iron ( $\mu$ mol/day)	output (g dry weight/day)	Cations <sup>b</sup> ( $\mu$ mol/g)	wet weight <sup>c</sup> (%)
control	$11 \pm 1$	$0.66 \pm 0.03$	$235 \pm 26$	$59.2 \pm 2.6$
heme	$34 \pm 1^*$	$0.83 \pm 0.02^*$	$806 \pm 24^*$	$83.9 \pm 0.4^\#$
protoporphyrin IX	$12 \pm 1$	$0.65 \pm 0.02$	$277 \pm 25$	$63.4 \pm 2.2$
ferric citrate	$37 \pm 2^*$	$0.67 \pm 0.03$	$171 \pm 17$	$51.6 \pm 2.8$
bilirubin	$10 \pm 1$	$0.64 \pm 0.03$	$259 \pm 19$	$62.1 \pm 1.9$

<sup>a</sup> Values are given as mean  $\pm$  SEM, (n=8). <sup>b</sup> Sodium, potassium and ammonia were measured as the major fecal cations. <sup>c</sup> Fecal wet weight percentage was calculated with the assumption that the total amount of sodium, potassium and ammonia and their counterions provided an osmolarity of 300 mosmol/L in feces (22). \* Significantly different from control group (p<0.05, LSD). # Significantly different from control group (p<0.05, Mann-Whitney U-test)

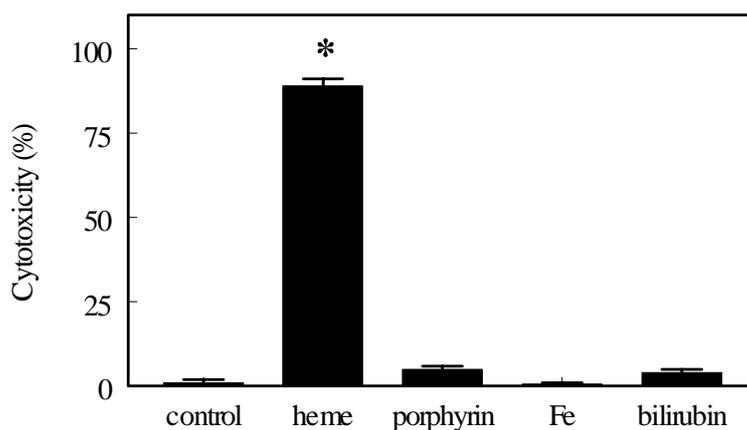


**Figure 1.** Effect of dietary tetrapyrroles and inorganic iron on proliferation of the colonic epithelium (mean  $\pm$  SEM,  $n=8$ ). Porphyrin: diet containing protoporphyrin IX. Fe: diet containing ferric citrate. \* Significantly different from control group ( $p<0.05$ , LSD).

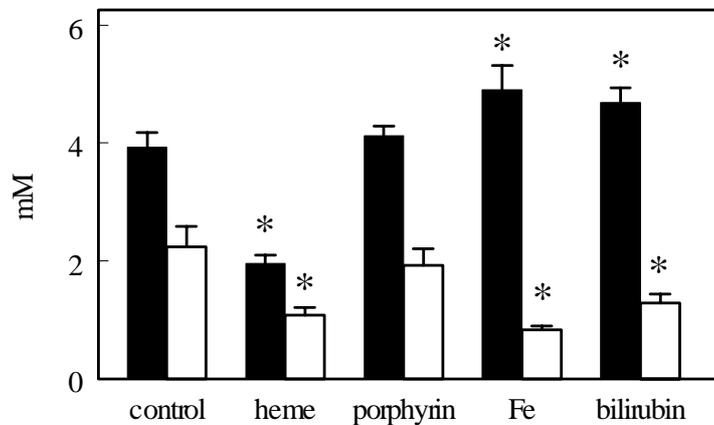


**Figure 2.** Dose-dependent cytotoxicity of fecal water of pooled samples of control (filled circles) and heme group (open circles) (mean  $\pm$  SEM,  $n=3$ ).

The colonic epithelium is mainly affected by water-soluble compounds in feces (26, 27). Therefore, cytotoxicity of fecal water was subsequently determined. First, the concentration dependence of cytotoxicity of fecal water of the heme and control group was tested. Figure 2 shows that colonic cytotoxicity was extremely high in the heme group: already

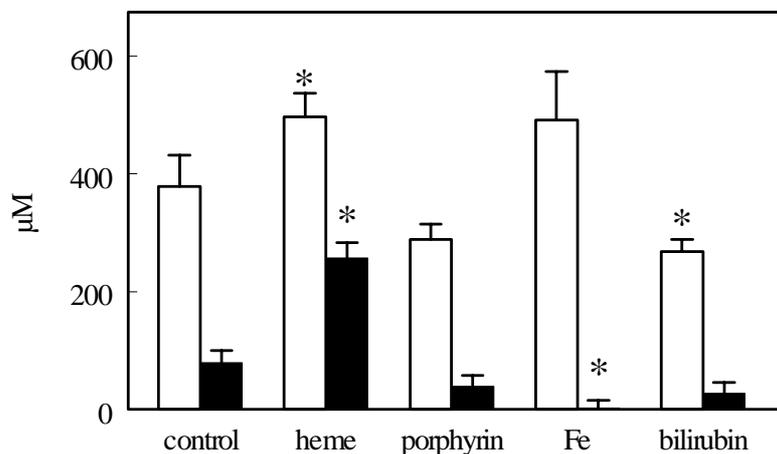


**Figure 3.** Cytotoxicity of 10  $\mu$ L fecal water of rats after feeding the experimental diets for 14 days (mean  $\pm$  SEM, n=8). Porphyrin: diet containing protoporphyrin IX. Fe: diet containing ferric citrate. \* Significantly different from control group (p<0.001, Mann-Whitney U-test).

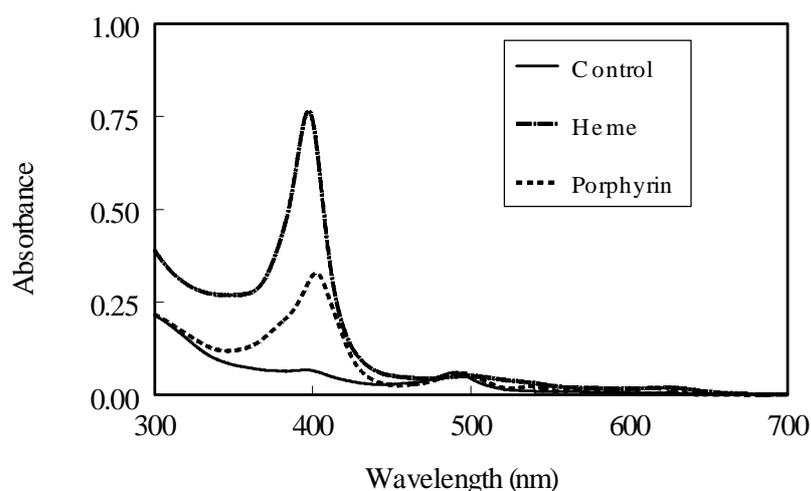


**Figure 4.** Concentrations of bile acids (filled bars) and fatty acids (open bars) in fecal water after rats were fed the experimental diets for 14 days (mean  $\pm$  SEM, n=8). Porphyrin: diet containing protoporphyrin IX. Fe: diet containing ferric citrate. \* Significantly different from control group (p<0.05, LSD).

10 $\mu$ L of fecal water were sufficient to lyse all erythrocytes. This implies that above 10  $\mu$ L, cytotoxicity of fecal water of the heme group would be underestimated. So, cytotoxicity of all fecal waters was tested using 10  $\mu$ L in our bioassay: the results are shown in Figure 3. Again, only the heme group showed increased cytotoxicity of fecal water, whereas all other experimental groups did not differ from control. As bile acids and fatty acids are important determinants of fecal water cytotoxicity (16), we tested whether their concentrations were increased by dietary heme. This was not the case, as less bile acids and fatty acids were present in fecal water of the heme group (Figure 4). Because we showed that about 90% of dietary heme reached the colon, we determined whether heme or heme-derived factors were solubilized in the aqueous phase. Figure 5 shows that, whereas both iron-supplemented groups had slightly elevated levels of total iron, only heme-iron increased the organic iron content of fecal water. In addition, Figure 6 shows that there was a concomitant large increase in the absorbance at 400 nm of the fecal water for the heme group and a moderate increase for the protoporphyrin group, while the spectra of the ferric citrate and the bilirubin groups were not different from the control. Together, these results suggest that heme was solubilized in the aqueous phase of the feces. Because heme has been shown previously to be lytic towards erythrocytes (17), we tested whether heme itself could be responsible for the extreme cytotoxicity observed in feces of the heme-fed rats. However, when we added 250  $\mu$ M of heme to fecal water of control rats, which is equivalent to the organic iron concentration of fecal water of the heme group, cytotoxicity was not enhanced under our experimental conditions (results not shown).

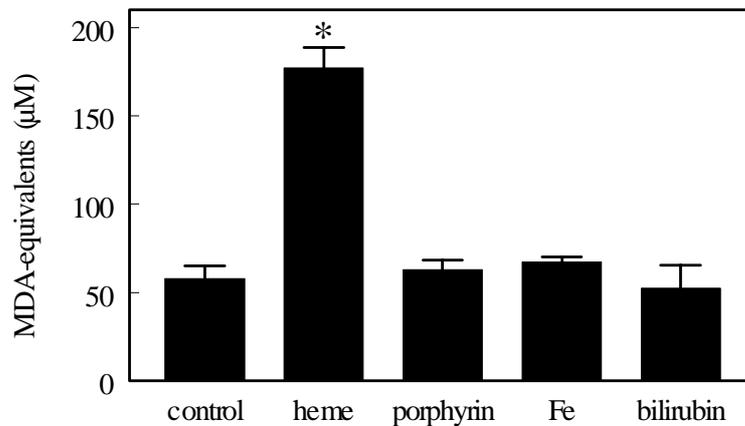


**Figure 5.** Concentration of total iron (open bars) and organic iron (filled bars) in fecal water (mean  $\pm$  SEM, n=8). Porphyrin: diet containing protoporphyrin IX. Fe: diet containing ferric citrate. \* Significantly different from control group (p<0.05, LSD).



**Figure 6.** UV-visible absorbance of acidified chloroform-methanol extracts of fecal water of the control, heme and protoporphyrin IX groups. Spectra of the ferric citrate and bilirubin group did not differ from the control spectrum.

Iron is thought to be a catalyst in the generation of oxygen radicals and lipid peroxidation products. Thus the question raised whether lipid peroxidation products mediated the observed detrimental effects of heme. Therefore we examined the reactivity of fecal water towards thiobarbituric acid (TBA). TBARS (TBA-reactive substances) were increased in the fecal water of heme-fed rats, but not in other treatment groups (Figure 7). To assure that the TBARS were not formed artificially during the preparation of the fecal water or during the TBARS-assay, heme was added to lyophilized feces of the control group and fecal water was prepared. No increase in TBARS was seen in the fecal water of the heme-supplemented control feces compared with control fecal water. In addition, reactivity of control fecal water towards thiobarbituric acid was not affected by addition of heme directly in the assay. Finally, to test whether oxygen radicals themselves might be responsible for the heme-induced cytotoxicity, we determined the dose-dependent cytotoxicity of fecal water of the heme group in an anaerobic cabinet and at ambient atmosphere. No significant differences were observed (results not shown). Thus, cytotoxicity of fecal water did not depend on the presence of oxygen.



**Figure 7.** Presence of TBARS in fecal water used as marker for luminal lipid peroxidation (mean  $\pm$  SEM,  $n=8$ ). Porphyrin: diet containing protoporphyrin IX. Fe: diet containing ferric citrate. \* Significantly different from control group ( $p<0.05$ , Mann-Whitney U-test).

## DISCUSSION

To our knowledge, this is the first study, which describes the effects of dietary heme iron on colonic cytotoxicity and epithelial proliferation in rats. We have shown that the apparent iron absorption (occurring in the small intestine) of the control group was about 12% of dietary intake and that additional uptake of iron from supplemental heme and ferric citrate was very low. Thus, the majority of dietary heme iron and iron from ferric citrate reaches the colon. From our results it is to be concluded that the unabsorbed heme iron, and not the iron by itself, is responsible for the detrimental effects in the colon observed in this study. The mild diarrhea (reflected by the increased output of cations) in the heme-fed rats probably reflects an impaired secretion or absorption capacity of the colon, caused by the high cytotoxicity of the colonic contents. Previous studies in our laboratory showed that diet-induced changes in cytotoxicity of fecal water highly correlated with colonic epithelial proliferation (16,26). Indeed, an increased cell turnover in the colonic mucosa, reflecting increased risk for colon cancer (28), accompanied the heme-induced cytotoxicity of fecal water.

Thus, ingestion of heme obviously leads to the formation of very cytotoxic compounds in the intestinal lumen. Our results may shed some light on the nature of this heme-induced cytotoxic factor. Figure 4, showing that the concentrations of bile acids and fatty acids were lower in fecal water of heme-fed rats compared with controls, suggests that these surfactants also were not responsible for the heme-induced cytotoxicity. This means that in heme-fed rats, an

additional, cytotoxic factor is formed and solubilized in fecal water. Figure 5 shows that the total iron content in fecal water was not different between the heme group and the ferric citrate group. This implies that total iron in fecal water is not related to cytotoxicity and hyperproliferation. However, the organic iron content of fecal water was much higher in the heme group than in other dietary groups. In addition, a sharp rise in absorbance at 400 nm was observed in fecal water of the heme group (Figure 6). Heme compounds show a specific absorption at 400 nm (Soretband). These two observations indicate that part of the organic iron in fecal water might be intact heme that has escaped intestinal absorption and metabolic conversion. Indeed, others have shown that part of dietary heme appears unmetabolized in feces (15, 29, 30). So the high cytotoxicity in the heme group could simply be caused by heme itself. However, addition of equimolar amounts of heme to fecal water of the control group did not enhance cytotoxicity. Well-known metabolites of heme in the gastrointestinal tract are protoporphyrin, inorganic iron (15, 29, 30) and bilirubin (18). No increase in cytotoxicity of fecal water or in epithelial proliferation was seen when these compounds were added to the diet. Thus, a role for these luminal-derived products as a cytotoxic factor can be excluded. Our observation that bilirubin is inert in our model contrasts with the work of Babbs et al., who suggested that iron, which acts as a catalyst in oxygen radical formation, is solubilized by linear tetrapyrroles, like bilirubin (11). We question this role for linear tetrapyrroles *in vivo*, because our spectrophotometric analyses show that bilirubin (which has an absorption maximum at 453 nm) is not solubilized in fecal water (Figure 6). Hence, it does not chelate soluble iron *in vivo*. Moreover, we showed that addition of bilirubin to the diet did not affect any luminal or mucosal parameter.

It is tempting to speculate that the formation of the cytotoxic compound may be due to the action of oxygen radicals. The increase in fecal TBARS suggests that these reactive oxygen species are indeed formed in the colonic lumen of heme-fed rats. However, these radicals do not seem to be directly involved in heme-induced cytotoxicity, as under both aerobic and anaerobic conditions, cytotoxicity of fecal water was the same. Due to the presence of thirteen unsaturated bonds, heme itself seems particularly vulnerable to attack by these highly reactive oxygen species. Future research is needed to elucidate the nature of the heme-induced cytotoxic factor. This should provide more insight into its formation as well as into the mechanism of its cytotoxic effects.

In this study, heme was used as a model compound for the consumption of red meat. In the literature, only a few experimental studies regarding the effect of beef on tumor development in rats are reported. Rats, injected with the experimental carcinogen dimethylhydrazine, developed more colon tumors when they were beef-fed (31). On the other hand, Lai et al. observed no effect of lean beef on formation of dimethylhydrazine-induced colon tumors (32). These contrasting results suggest that the effect of the carcinogen may be dependent on the dietary background. McIntosh et al. (31) used a high-fat diet (20% fat, w/w), which may promote colon tumor formation, induced by carcinogens (33), compared with low fat diets, as used by Lai et al. (6.25% fat, w/w) (32). This implies that if dimethylhydrazine-induced tumor formation is mediated by heme-iron from beef, these heme effects can be

modulated by other dietary factors. We think that our heme model is very well suited to study possible interactions of heme with other dietary ingredients, for instance the effect of poorly absorbed nutrients, like calcium, fiber or some antioxidants, which are epidemiologically associated with a decrease in colon cancer risk.

In conclusion, dietary heme induces fecal cytotoxicity and hyperproliferation of the colonic mucosa in rats. Consumption of red meat, but not of white meat, is associated with a high risk for colon cancer. As the heme content of red meat is tenfold higher than that of white meat (15), we suggest that the association between red meat consumption and colon cancer may be due to its high content of heme iron. As formation of the highly cytotoxic factor in feces was specific for dietary heme, identification and quantification of this compound in fecal water would provide further insight into the molecular mechanism of the relationship between red meat consumption and colon cancer, observed in epidemiological studies.

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