

# Red Meat and Colon Cancer: The Cytotoxic and Hyperproliferative Effects of Dietary Heme<sup>1</sup>

Aloys L. A. Sesink,<sup>2</sup> Denise S. M. L. Termont, Jan H. Kleibeuker, and Roelof Van der Meer

Wageningen Centre for Food Sciences, Netherlands Institute for Dairy Research Food Research, Section Nutrition and Health, 6710 BA Ede, the Netherlands [A. L. A. S., D. S. M. L. T., R. V. d. M.], and Department of Gastroenterology, University Hospital, Groningen, the Netherlands [J. H. K.]

## ABSTRACT

The 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 hemin (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 [<sup>3</sup>H]thymidine incorporation into colonic mucosa.

The colonic epithelial proliferation in heme-fed rats was significantly increased compared to control rats [ $55.2 \pm 5.8$  versus  $32.6 \pm 6.3$  dpm/ $\mu\text{g}$  DNA (mean  $\pm$  SE);  $P < 0.05$ ]. The fecal water of the heme group was highly cytotoxic compared to the controls ( $90 \pm 2\%$  versus  $2 \pm 1\%$ ;  $P < 0.001$ ), although the concentrations of cytotoxic bile acids and fatty acids were significantly lower. Organic iron was significantly increased compared to the controls ( $257 \pm 26$  versus  $80 \pm 21$   $\mu\text{M}$ ;  $P < 0.001$ ). Spectrophotometric analyses suggest that this organic iron is heme-associated. Thiobarbituric acid-reactive substances were greatly increased in the fecal water of heme-fed rats compared to the controls ( $177 \pm 12$  versus  $59 \pm 7$   $\mu\text{M}$ ;  $P < 0.05$ ). Heme itself could not account for the increased cytotoxicity because the addition of heme to the fecal water of the control group, which was equimolar to the organic iron content of the fecal water of the heme group, did not influence the 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 the 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. Every year in the United States, 110,000 new cases are diagnosed, 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). Although 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, which is 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 because 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. Although proven to be mutagenic *in vitro*, the 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 the 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  $\sim 1.5$   $\mu\text{mol}$  heme/g dry weight; Ref. 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, such as bile acids and fatty acids, can cause epithelial damage, resulting in a compensatory epithelial hyperproliferation (16). Furthermore, it was shown that heme is cytotoxic toward 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, because heme is an important iron carrier, ferric citrate was also included in the study.

Received 4/16/99; accepted 9/22/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported by Grant 904–62–167 from The Netherlands Organization for Scientific Research, Medical Sciences. An abstract of this study was presented at the Digestive Disease Week 1998.

<sup>2</sup> To whom requests for reprints should be addressed, at Wageningen Centre for Food Sciences, NIZO Food Research, Section Nutrition and Health, P. O. Box 20, 6710 BA Ede, the Netherlands. Phone: 31-318-659621; Fax: 31-318-650400; E-mail: Sesink@nizo.nl.

## 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) with a mean body weight of  $325 \pm 2$  g were housed individually in metabolic cages in a room with controlled temperature ( $22^{\circ}\text{C}$ – $24^{\circ}\text{C}$ ), relative humidity (50–60%), and light/dark cycle (lights on from 6 a.m. to 6 p.m.). During 2 weeks, five groups of eight rats were fed purified diets. The control diet contained per kilogram, 200 g of casein, 617 g of dextrose, 115 g of fat (82% palm oil and 18% corn oil), 20 g of cellulose, and 20 mmol of 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), 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, nonfasted rats received injections i.p. of [methyl- $^3\text{H}$ ]thymidine (specific activity, 25 Ci/mmol; dose,  $100 \mu\text{Ci/kg}$  body weight; Amersham International, Buckinghamshire, United Kingdom) 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). The scrapings were analyzed as described previously (20). For protein determinations,  $100 \mu\text{l}$  of homogenate were diluted 10-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 SDS (50 mg/ml) in 100 mM NaOH. Protein was quantified according to Smith *et al.* (21) using the 2 Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL), with BSA as the 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 water bath 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 osmolality was measured (Osmomat 030-D, Gonotec, Berlin, Germany). If osmolality differed from 300 mOsmol/L, another portion of freeze dried feces was reconstituted with double-distilled water, and the percentage dry weight was adjusted to obtain fecal water with an osmolality of 300 mOsmol/L. Fecal water was stored at  $-20^{\circ}\text{C}$  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 heme groups was determined by supplementing increasing volumes of fecal water with saline to a total volume of  $80 \mu\text{l}$ . After preincubation for 5 min at  $37^{\circ}\text{C}$ ,  $20 \mu\text{l}$  of a washed human erythrocyte suspension were added (final hematocrit, 5%) and incubated for 15 min at  $37^{\circ}\text{C}$ . Cytotoxicity was measured as described before (22). Subsequently, differences between experimental groups were quantified by testing the cytotoxicity of  $10 \mu\text{l}$  of fecal water. Finally, to test whether fecal cytotoxicity of heme-fed rats was dependent on oxygen, the cytotoxicity of increasing volumes of fecal water of the heme group was determined as described above in an anaerobic cabinet (Coy Laboratory products, Ann Arbor, MI). Simultaneously, equal amounts were tested at ambient atmosphere.

**Total Feces Analyses.** For total iron determination, feces were dry-ashed for 8 h at  $550^{\circ}\text{C}$  (Heraeus, Eurotherm 815, Boom Meppel, the Netherlands), followed by destruction (20 min;  $180^{\circ}\text{C}$ ) with  $500 \mu\text{l}$  of perchloric acid (70%) and  $100 \mu\text{l}$  of  $\text{H}_2\text{O}_2$  (30%). After dilution in double-distilled water, iron was measured using an atomic absorption spectrometer (Perkin-Elmer, model

1100, Norwalk, CT). The 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 the determination of the ammonia content, feces were incubated for 15 min at  $37^{\circ}\text{C}$  in 5% perchloric acid. Samples were centrifuged for 2 min at 14,000 g, and ammonia was measured in the supernatant by using the Sigma Urea Nitrogen Kit (Sigma Diagnostics, No. 640, St. Louis, MO) and omitting the incubation step with urease. The percentage of water in the feces was calculated assuming that the total amount of sodium, potassium, ammonia, and their negatively charged counterions provided an osmolality of 300 mOsmol/L in feces (23).

**Analyses of Fecal Water.** To determine the total iron in fecal water, samples were treated as described for feces except that the dry-ashing step was omitted, and  $50 \mu\text{l}$  of perchloric acid and  $20 \mu\text{l}$   $\text{H}_2\text{O}_2$  were used for destruction. For inorganic iron determination, fecal water was incubated in 10% trichloroacetic acid for 20 min at  $90^{\circ}\text{C}$  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, the recovery of added ferric citrate as inorganic iron was  $97\% \pm 2\%$ , and the recovery of hemin as organic iron was  $92\% \pm 7\%$ . For the determination of free fatty acids and bile acids in fecal water, acidified fecal water (final HCl concentration, 1M) was extracted three times with five 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).

To evaluate lipid peroxidative processes in the lumen, TBARSs<sup>3</sup> in fecal water were measured according to Ohkawa *et al.* (25). Briefly, fecal water was diluted 10-fold with double-distilled water, and  $100 \mu\text{l}$  of this diluted fecal water was mixed with  $100 \mu\text{l}$  of 8.1% SDS and  $1000 \mu\text{l}$  of 0.5% TBA in 10% acetic acid (pH 3.5). For blanks, TBA was omitted from the assay. After heating for 60 min at  $95^{\circ}\text{C}$ , TBARSs were extracted with 1.2 ml *n*-butyl alcohol. The absorbance of this butanol extract was measured at 532 nm. The amount of TBARSs was calculated as malondialdehyde equivalents by using 1,1,3,3-tetramethoxypropane as the standard.

**Statistics.** Results are presented as means  $\pm$  SE ( $n = 8$ ). 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 ANOVA was performed, followed by Fisher's LSD test (two-sided) to test for significant differences between means of dietary treatments and control diet. When data of groups showed unequal variances, the 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 absorp-

<sup>3</sup> TBARS, thiobarbituric acid-reactive substance; TBA, thiobarbituric acid; LSD, least significant difference.

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

Diet <sup>b</sup>	Total iron ( $\mu\text{mol}/\text{day}$ )	Output (g dry weight/day)	Cations <sup>c</sup> ( $\mu\text{mol}/\text{g}$ )	Wet weight <sup>d</sup> (%)
Control	11 $\pm$ 1	0.66 $\pm$ 0.03	235 $\pm$ 26	59.2 $\pm$ 2.6
Heme	34 $\pm$ 1 <sup>e</sup>	0.83 $\pm$ 0.02 <sup>e</sup>	806 $\pm$ 24 <sup>e</sup>	83.9 $\pm$ 0.4 <sup>f</sup>
Protoporphyrin IX	12 $\pm$ 1	0.65 $\pm$ 0.02	277 $\pm$ 25	63.4 $\pm$ 2.2
Ferric citrate	37 $\pm$ 2 <sup>e</sup>	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  $\pm$  SE ( $n = 8$ ).

<sup>b</sup> Rats were fed diets for 14 days. Experimental diets were supplemented with 1.3  $\mu\text{mol}/\text{g}$  of heme (ferriheme), protoporphyrin IX, bilirubin, or ferric citrate. Feces were collected at day 11–14 of the experiment and frozen immediately.

<sup>c</sup> Sodium, potassium, and ammonia were measured as the major fecal cations.

<sup>d</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).

<sup>e</sup> Significantly different from control group ( $p < 0.05$ , LSD).

<sup>f</sup> Significantly different from control group ( $p < 0.05$ , Mann-Whitney  $U$ -test).

tion from the control diet was  $\sim 12\%$ . 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, the softening of feces of heme-fed rats was observed, whereas feces from other experimental groups had an 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, the 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. Fig. 1 shows that the heme diet significantly increased proliferation compared with the control diet, whereas proliferation was not affected by the other dietary treatments. DNA and protein content did not differ among the groups (DNA,  $874 \pm 83 \mu\text{g}/\text{scraping}$ ; protein,  $9.1 \pm 1.0 \text{ mg}/\text{scraping}$ ).

The colonic epithelium is mainly affected by water-soluble compounds in feces (26, 27). Therefore, the cytotoxicity of the fecal water was subsequently determined. First, the concentration dependence of the cytotoxicity of the fecal water of the heme and control groups was tested. Fig. 2 shows that the colonic cytotoxicity was extremely high in the heme group: already 10  $\mu\text{l}$  of fecal water were sufficient to lyse all erythrocytes. This implies that at  $> 10 \mu\text{l}$ , the cytotoxicity of the fecal water of the heme group would be underestimated. Therefore, the cytotoxicity of all fecal waters was tested using 10  $\mu\text{l}$  in our bioassay; the results are shown in Fig. 3. Again, only the heme group

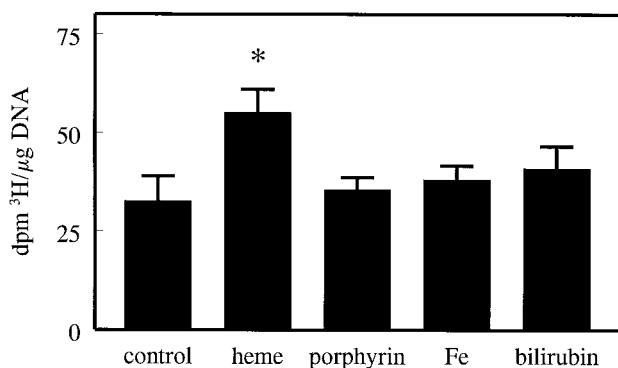


Fig. 1. The effect of dietary tetrapyrroles and inorganic iron on the proliferation of the colonic epithelium (dpm [<sup>3</sup>H]/ $\mu\text{g}$  DNA). Porphyrin, diet containing protoporphyrin IX. Fe, diet containing ferric citrate. \*, significantly different from control group ( $P < 0.05$ , LSD). Bars, SE.

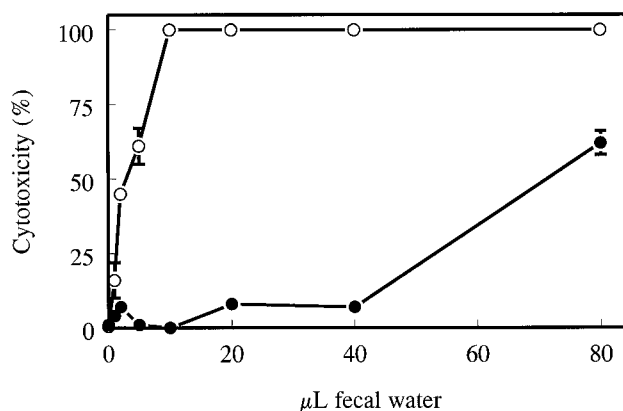


Fig. 2. The dose-dependent cytotoxicity of the fecal water of the pooled samples of control (solid circles) and the heme group (open circles). Each point represents the mean of three measurements. Bars, SE.

showed increased cytotoxicity of fecal water, whereas all other experimental groups did not differ from the control. Because 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 because fewer bile acids and fatty acids were present in the fecal water of the heme group (Fig. 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. Fig. 5 shows that whereas both iron-supplemented groups had slightly elevated levels of total iron, only heme iron increased the organic iron content of the fecal water. In addition, Fig. 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, whereas 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 toward erythrocytes (17), we tested whether heme itself could be responsible for the extreme cytotoxicity observed in the feces of the heme-fed rats. However, when we added 250  $\mu\text{M}$  of heme to the fecal water of the control rats, which is equivalent to the organic iron concentration of fecal water of the heme group, the cytotoxicity was not enhanced under our experimental conditions (results not shown).

Iron is thought to be a catalyst in the generation of oxygen radicals and lipid peroxidation products. Thus, the question was raised as to whether lipid peroxidation products mediated the observed detrimental effects of heme. Therefore, we examined the reactivity of fecal water toward TBA. TBARSs were increased in the fecal water of heme-fed rats but not in other treatment groups (Fig. 7). To assure that

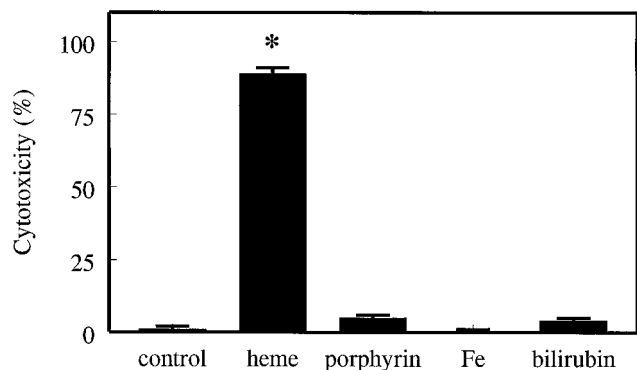


Fig. 3. The cytotoxicity of 10  $\mu$ l of fecal water of rats after feeding the experimental diets for 14 days. *Porphyrin*, diet containing protoporphyrin IX. *Fe*, diet containing ferric citrate. \*, significantly different from the control group ( $P < 0.001$ , Mann-Whitney *U* test). Bars, SE.

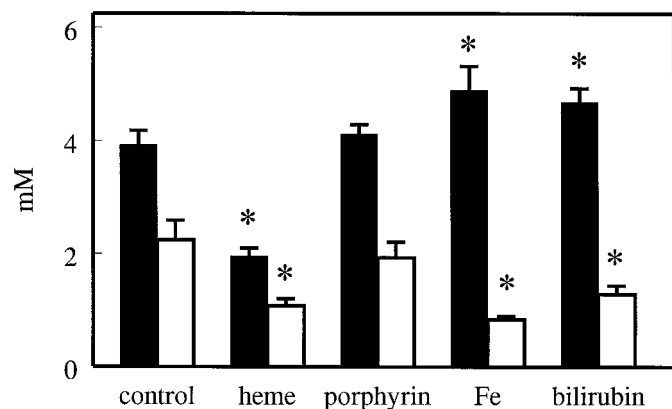


Fig. 4. The concentrations of bile acids (solid bars) and fatty acids (open bars) in fecal water after rats were fed the experimental diets for 14 days. *Porphyrin*, diet containing protoporphyrin IX. *Fe*, diet containing ferric citrate. \*, significantly different from the control group ( $P < 0.05$ , LSD). Bars, SE.

the TBARSs 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 TBARSs was seen in the fecal water of the heme-supplemented control feces compared with the control fecal water. In addition, the reactivity of the control fecal water toward TBA was not affected by the 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 the fecal water of the heme group in an anaerobic cabinet and at ambient atmosphere. No significant differences were observed (results not shown). Thus, the cytotoxicity of fecal water did not depend on the presence of oxygen.

**DISCUSSION**

To our knowledge, this is the first study that 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 the 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 the cytotoxicity of fecal water highly correlated with colonic epithelial proliferation (16, 26). Indeed, an increased cell turnover in the colonic mucosa reflecting an increased risk for colon cancer (28) accompanied the heme-induced cytotoxicity of fecal water.

Thus, the 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. Fig. 4, which shows 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. Fig. 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 the 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 the other dietary groups. In addition, a sharp rise in absorbance at 400 nm was observed in the fecal water of the heme group (Fig. 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 the dietary heme appears

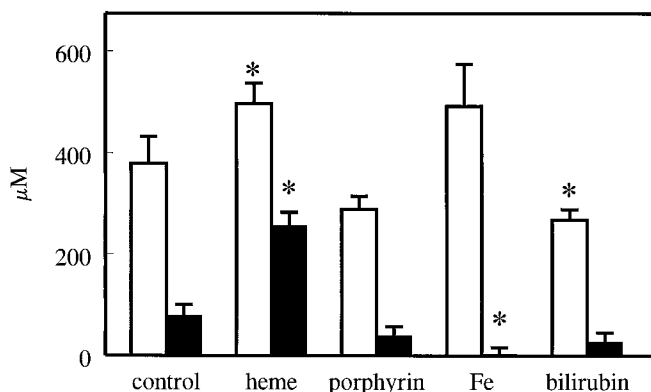


Fig. 5. The concentration of total iron (open bars) and organic iron (solid bars) in fecal water ( $\mu$ M). *Porphyrin*, diet containing protoporphyrin IX. *Fe*, diet containing ferric citrate. \*, significantly different from the control group ( $P < 0.05$ , LSD). Bars, SE.

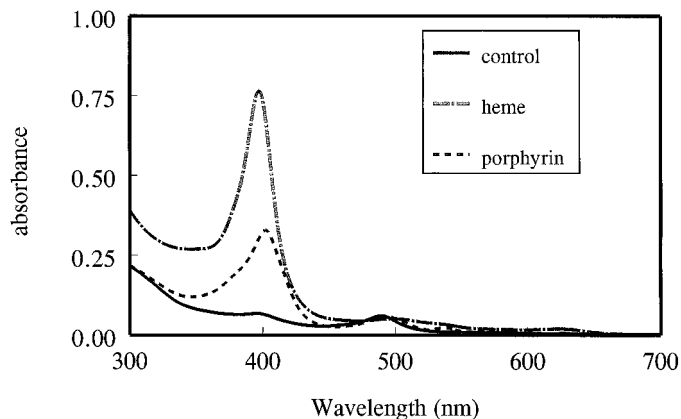


Fig. 6. UV-visible absorbance of acidified chloroform-methanol extracts of the fecal water of the control, heme, and protoporphyrin IX groups. The spectra of the ferric citrate and bilirubin group did not differ from the control spectrum.

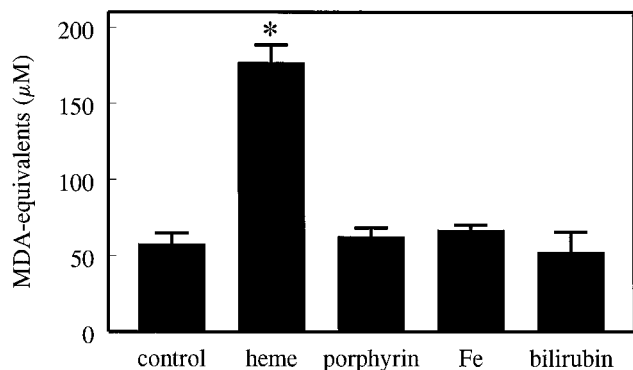


Fig. 7. The presence of TBARSs (micromolar malondialdehyde equivalents) in fecal water was used as a marker for luminal lipid peroxidation. *Porphyrin*, diet containing protoporphyrin IX. *Fe*, diet containing ferric citrate. \*, significantly different from the control group ( $P < 0.05$ , Mann-Whitney  $U$  test). Bars, SE.

unmetabolized in feces (15, 29, 30). So the high cytotoxicity in the heme group could simply be caused by the heme itself. However, the addition of equimolar amounts of heme to fecal water of the control group did not enhance the cytotoxicity. Well-known metabolites of heme in the gastrointestinal tract are protoporphyrin, inorganic iron (15, 29, 30), and bilirubin (18). No increase in the cytotoxicity of the 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 (11) who suggested that iron, which acts as a catalyst in oxygen radical formation, is solubilized by linear tetrapyrroles like bilirubin. 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 (Fig. 6). Hence, it does not chelate soluble iron *in vivo*. Moreover, we showed that the 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 TBARSs 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 because under both aerobic and anaerobic conditions, the 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 who received injections of the experimental carcinogen dimethylhydrazine developed more colon tumors when they were beef-fed (31). On the other hand, Lai *et al.* (32) observed no effect of lean beef on the formation of dimethylhydrazine-induced colon tumors. 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.* (Ref. 32; 6.25% fat, w/w). 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. Because the heme content of red meat is 10-fold 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. Because the 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.

## ACKNOWLEDGMENTS

We thank Mischa Wissink (NIZO Food Research), Bert Weijers, and Maria Faassen-Peters (Small Animal Research Center, Wageningen Agricultural University, Wageningen, the Netherlands) for their skillful biotechnical assistance.

## REFERENCES

- Potter, J. D. Colorectal cancer: epidemiology. *Encycl. Cancer*, 1: 441–450, 1997.
- Fearon, E. R., and Jones, P. A. Progressing toward a molecular description of colorectal cancer development. *FASEB J.*, 6: 2783–2790, 1992.
- MacLennan, R. Diet and colorectal cancer. *Int. J. Cancer*, (Suppl. 10): 10–12, 1997.
- Armstrong, B., and Doll, R. Environmental factors and cancer incidence and mortality in different countries with special reference to dietary practices. *Int. J. Cancer*, 15: 617–631, 1975.
- Willet, W. C., Stampfer, M. J., Colditz, G. A., Rosner, B. A., and Speizer, F. E. Relation of meat, fat and fiber intake to the risk of colon cancer in a prospective study among women. *N. Engl. J. Med.*, 323: 1664–1672, 1990.
- Giovannucci, E., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Ascherio, A., and Willet, W. C. Intake of fat, meat and fiber in relation to risk of colon cancer in men. *Cancer Res.*, 54: 2390–2397, 1994.
- Bingham, S. A. Epidemiology and mechanisms relating diet to risk of colorectal cancer. *Nutr. Res. Rev.*, 9: 197–239, 1996.
- Sinha, R., Rothman, N., Brown, E. D., Salmon, C. P., Knize, M. G., Swanson, C. A., Rossi, S. C., Mark, S. D., Levander, O. A., and Felton, J. S. High concentrations of the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) occur in chicken but are dependent on the cooking method. *Cancer Res.*, 55: 4516–4519, 1995.
- Giovannucci, E., and Goldin, B. The role of fat, fatty acids, and total energy intake in the etiology of human colon cancer. *Am. J. Clin. Nutr.*, 66 (Suppl. 6): 1564S–1571S, 1997.
- Graf, E., and Eaton, J. W. Dietary suppression of colonic cancer—fiber or phytate? *Cancer (Phila.)*, 56: 717–718, 1985.
- Babbs, C. F. Free radicals and the etiology of colon cancer. *Free Radic. Biol. Med.*, 8: 191–200, 1990.
- Chin, J., and Carpenter, C. E. Effects of dietary fat type and iron level on *in vivo* lipid peroxidation in rat colon. *Nutr. Res.*, 17: 1381–1389, 1997.
- Rimbach, G., Markant, A., Most, E., and Pallauf, J. Liver and colon oxidant status in growing rats fed increasing levels of dietary iron. *J. Trace Elem. Med. Biol.*, 11: 99–104, 1997.
- Stimson, J., and Fischer, J. G. Iron intake affects lipid peroxidation and glutathione peroxidase activity of distal colonic mucosa. *Nutr. Res.*, 17: 1683–1691, 1997.
- Schwartz, S., and Ellefson, M. Quantitative fecal recovery of ingested hemoglobin-heme in blood: comparisons by hemouquant assay with ingested meat and fish. *Gastroenterology*, 89: 19–26, 1985.
- Van der Meer, R., Lapré, J. A., Govers, M. J. A. P., and Kleibeuker, J. H. Mechanisms of the intestinal effects of dietary fats and milk products on colon carcinogenesis. *Cancer Lett.*, 114: 75–83, 1997.
- Kirschner-Zilber, I., Rabizadeh, E., and Shaklai, N. The interaction of hemin and bilirubin with the human red blood cell membrane. *Biochim. Biophys. Acta*, 690: 20–30, 1982.
- Rosenberg, D. W., and Kappas, A. Characterization of heme oxygenase in the small intestinal epithelium. *Arch. Biochem. Biophys.*, 274: 471–480, 1989.
- Reeves, P. G., Nielsen, F. H., and Fahey, G. C. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A diet. *J. Nutr.*, 123: 1939–1951, 1993.
- Govers, M. J. A. P., Termont, D. S. M. L., and Van der Meer, R. Mechanism of the antiproliferative effect of milk mineral and other calcium supplements on colonic epithelium. *Cancer Res.*, 54: 95–100, 1994.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goekce, M. N., Olson, B. J., and Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, 150: 76–85, 1985.

22. Govers, M. J. A. P., Termont, D. S. M. L., Lapré, J. A., Kleibeuker, J. H., Vonk, R. J., and Van der Meer, R. Calcium in milk products precipitates intestinal fatty acids and secondary bile acids and thus inhibits colonic cytotoxicity in humans. *Cancer Res.*, *56*: 3270–3275, 1996.
23. Fine, K. D., Krejs, G. J., and Fordtran, J. S. Diarrhea. In: Sleisinger M. H. and Fordtran J. S. (eds.), *Gastrointestinal disease: pathophysiology, diagnosis and management*, pp. 1043–1072. Philadelphia: W. B. Saunders Company, 1993.
24. Bligh, E. G., and Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, *37*: 911–917, 1959.
25. Ohkawa, H., Ohishi, N., and Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, *95*: 351–358, 1979.
26. Lapré, J. A., De Vries, H. T., Koeman, J. H., and Van der Meer, R. The anti-proliferative effect of dietary calcium on colonic epithelium is mediated by luminal surfactants and dependent on the type of dietary fat. *Cancer Res.*, *53*: 784–789, 1993.
27. Rafter, J. J., Child, P., Anderson, A. M., Alder, R., Eng, V., and Bruce, R. Cellular toxicity of fecal water depends on diet. *Am. J. Clin. Nutr.*, *45*: 559–563, 1987.
28. Lipkin, M. Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Res.*, *48*: 235–245, 1988.
29. Rose, I. S., Young, G. P., St. John, D. J. B., Deacon, M. C., Blake, D., and Henderson, R. W. Effect of ingestion of hemoproteins on fecal excretion of hemes and porphyrins. *Clin. Chem.*, *35*: 2290–2296, 1989.
30. Young, G. P., St. John, D. J. B., Rose, I. S., and Blake, D. Haem in the gut. II. Faecal excretion of haem and haem-derived porphyrins and their detection. *J. Gastroenterol. Hepatol.*, *5*: 194–203, 1990.
31. McIntosh, G. H., Regester, G. O., Le Leu, R. K., Royle, P. J., and Smithers, G. W. Dairy proteins protect against dimethylhydrazine-induced intestinal cancer in rats. *J. Nutr.*, *125*: 809–816, 1995.
32. Lai, C., Dunn, D. M., Miller, M. F., and Pence, B. C. Non-promoting effects of iron from beef in the rat colon carcinogenesis model. *Cancer Lett.*, *112*: 87–91, 1997.
33. Ma, Q., Hoper, M., Halliday, I., and Rowlands, B. J. Diet and experimental colorectal cancer. *Nutr. Res.*, *16*: 413–426, 1996.

# Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

## Red Meat and Colon Cancer: The Cytotoxic and Hyperproliferative Effects of Dietary Heme

Aloys L. A. Sesink, Denise S. M. L. Termont, Jan H. Kleibeuker, et al.

*Cancer Res* 1999;59:5704-5709.

**Updated version** Access the most recent version of this article at:  
<http://cancerres.aacrjournals.org/content/59/22/5704>

**Cited articles** This article cites 28 articles, 9 of which you can access for free at:  
<http://cancerres.aacrjournals.org/content/59/22/5704.full#ref-list-1>

**Citing articles** This article has been cited by 26 HighWire-hosted articles. Access the articles at:  
<http://cancerres.aacrjournals.org/content/59/22/5704.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://cancerres.aacrjournals.org/content/59/22/5704>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.