

## Green vegetables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon

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**Diets high in red meat and low in green vegetables are associated with increased colon cancer risk. This association might be partly due to the haem content of red meat. In rats, dietary haem is metabolized in the gut to a cytotoxic factor that increases colonic cytotoxicity and epithelial proliferation. Green vegetables contain chlorophyll, a magnesium porphyrin structurally analogous to haem. We studied whether green vegetables inhibit the unfavourable colonic effects of haem. First, rats were fed a purified control diet or purified diets supplemented with 0.5 mmol haem/kg, spinach (chlorophyll concentration 1.2 mmol/kg) or haem plus spinach ( $n = 8$ /group) for 14 days. In a second experiment we also studied a group that received haem plus purified chlorophyll (1.2 mmol/kg). Cytotoxicity of faecal water was determined with a bioassay and colonic epithelial cell proliferation was quantified *in vivo* by [methyl-<sup>3</sup>H]thymidine incorporation into newly synthesized DNA. Exfoliation of colonocytes was measured as the amount of rat DNA in faeces. In both studies haem increased cytotoxicity of the colonic contents ~8-fold and proliferation of the colonocytes almost 2-fold. Spinach or an equimolar amount of chlorophyll supplement in the haem diet inhibited these haem effects completely. Haem clearly inhibited exfoliation of colonocytes, an effect counteracted by spinach and chlorophyll. Finally, size exclusion chromatography showed that chlorophyll prevented formation of the cytotoxic haem metabolite. We conclude that green vegetables may decrease colon cancer risk because chlorophyll prevents the detrimental, cytotoxic and hyperproliferative colonic effects of dietary haem.**

### Introduction

Colon cancer is one of the leading causes of cancer death in Western societies. For the USA in 2003 nearly 110 000 new cases and almost 48 000 deaths were estimated (1). Incidence rates of colon cancer vary 20-fold between high and low risk countries (2). Migrant and other epidemiological studies indicate that this variation is due to environmental factors, with

diet as a major determinant (3). Diets high in red and processed meat are especially associated with a moderately increased risk for colon cancer (4). In contrast, diets high in white meat (poultry, fish) are not associated with an increased risk (5). The mechanism explaining the specific risk-enhancing effect of red meat is not precisely known. Based on mutational analysis of colon cancers, Kinzler and Vogelstein (6) argued that dietary factors that lead to colon cancer are probably not mutagens but rather luminal irritants that damage colonic epithelial cells (7). This damage triggers a compensatory epithelial hyperproliferation, which increases the risk of endogenous mutations in tumour suppressor and oncogenes. Clonal accumulation of these endogenous mutations may eventually result in the adenoma–carcinoma sequence of colon carcinogenesis (8). For these reasons, Sesink *et al.* (9) hypothesized that haem, the iron porphyrin pigment of red meat, might be an important dietary risk factor. They argued that haem may better explain the differential effects of red versus white meat on colon cancer risk, instead of earlier proposed meat-associated mutagens such as heterocyclic amines (10). Sesink *et al.* (9) showed in rat studies that dietary haem enhanced cytotoxicity of the faecal water. This was not mediated by well-known surfactants like bile acids or fatty acids, suggesting involvement of a haem-induced cytotoxic metabolite. This enhanced cytotoxicity implies an increased exposure of the colonic epithelial cells to luminal irritants, resulting in colonic epithelial hyperproliferation. As mentioned above, hyperproliferation could increase the risk of endogenous mutations in cell turnover genes and is therefore considered an early risk marker for colon cancer (11). This is supported by the results of clinical studies where subjects at high risk of colon cancer showed a higher proliferative activity compared with controls (12).

A large number of epidemiological studies indicate that vegetables protect against colon cancer, especially green and raw vegetables (13,14). The aim of this study was to investigate whether the protective effect of vegetables is due to inhibition of haem-induced colonic cytotoxicity and hyperproliferation of epithelial cells. We hypothesized that this protection could be due to the high chlorophyll content of green vegetables. Chlorophyll is a phytol-esterified magnesium porphyrin and thus a structural analogue of haem. Therefore, it might compete with haem for solubilization in the gastrointestinal tract and thus prevent the formation of cytotoxic haem metabolites. Subsequently, this may prevent haem-induced hyperproliferative effects. We first examined the effect of the green vegetable spinach and, second, whether the effect of spinach can be mimicked by an equimolar concentration of chlorophyll.

### Materials and methods

#### *Animals and diets*

The experimental protocols were approved by the animal welfare committee of Wageningen University and Research Centre. Approximately 8-week-old

**Abbreviations:** ANOVA, one-way analysis of variance; ICP-AES, inductive coupled plasma absorption emission spectrophotometer.

Table I. Composition of the experimental diets (g/kg)

| Ingredient                            | Study 1 |       |         |                   | Study 2 |       |                   |                       |
|---------------------------------------|---------|-------|---------|-------------------|---------|-------|-------------------|-----------------------|
|                                       | Control | Haem  | Spinach | Haem plus spinach | Control | Haem  | Haem plus spinach | Haem plus chlorophyll |
| Acid casein <sup>a</sup>              | 200     | 200   | 176     | 176               | 200     | 200   | 176               | 200                   |
| Dextrose <sup>a</sup>                 | 532     | 532   | 506     | 506               | 532     | 532   | 506               | 532                   |
| Palm fat                              | 160     | 160   | 160     | 160               | 148     | 148   | 148               | 148                   |
| Palm oil                              |         |       |         |                   | 12      | 12    | 12                |                       |
| Corn oil                              | 40      | 40    | 40      | 40                | 40      | 40    | 40                | 40                    |
| Cellulose <sup>a</sup>                | 20      | 20    |         |                   | 20      | 20    |                   | 20                    |
| Haem                                  |         | 0.326 |         | 0.326             |         | 0.326 | 0.326             | 0.326                 |
| Spinach                               |         |       | 82      | 82                |         |       | 82                |                       |
| Natural chlorophyll                   |         |       |         |                   |         |       |                   | 12                    |
| CaHPO <sub>4</sub> ·2H <sub>2</sub> O | 3.44    | 3.44  |         |                   | 3.44    | 3.44  |                   | 3.44                  |
| Mineral mix <sup>a</sup>              | 35      | 35    | 26.3    | 26.3              | 35      | 35    | 26.3              | 35                    |
| Vitamin mix                           | 10      | 10    | 10      | 10                | 10      | 10    | 10                | 10                    |

The composition of the vitamin and mineral mixtures is according to the recommendations of the American Institute of Nutrition 1993, except that calcium was omitted. In addition, tripotassium citrate was added instead of KH<sub>2</sub>PO<sub>4</sub> and choline was added as choline chloride.

<sup>a</sup>Protein, carbohydrate, cellulose, calcium and potassium were adjusted for their content in spinach.

outbred male Wistar rats (WU, Harlan, Horst, The Netherlands) were housed individually in metabolic cages in a room with controlled temperature (±20°C), relative humidity (50–60%) and light/dark cycle (lights on 6 a.m. to 6 p.m.). Animals were acclimatized to housing conditions for 5 days before the start of the experiment.

In the first study we tested whether spinach prevents the cytotoxic and hyperproliferative effects of dietary haem. During 2 weeks four groups of eight rats were fed purified diets. The compositions of the diets are given in Table I. The haem and haem plus spinach diets were supplemented with 0.5 mmol haem/kg diet (Sigma-Aldrich, St Louis, MO). The iron content of the haem was analyzed with an inductive coupled plasma absorption emission spectrophotometer (ICP-AES) (Varian, Mulgrave, Australia), giving a purity of >90%.

The spinach diets contained 82 g/kg powdered freeze-dried spinach at the expense of acid casein, dextrose and cellulose, with a final chlorophyll concentration of ~1.2 mmol chlorophyll/kg diet. We measured the chlorophyll concentration of spinach (Iglo, 's-Hertogenbosch, The Netherlands) after five successive extractions with acetone/water (80:20 v/v) by comparing the absorption spectra with standard chlorophyll solutions (Sigma) in a spectrophotometer (Lambda 2; Perkin Elmer, Norwalk, CT). Calcium was added as calcium phosphate (CaHPO<sub>4</sub>·2H<sub>2</sub>O) (Fluka Chemie, Buchs, Switzerland). Vitamins and minerals other than calcium were added to all diets in similar concentrations according to the recommendations of the American Institute of Nutrition 1993 (15).

In a second study, using a similar experimental design as the first, we tested whether the protection by spinach against haem-induced hyperproliferation might be due to its chlorophyll content. The chlorophyll plus haem diet was prepared as the haem diet (Table I) with the addition of 12 g natural chlorophyll in palm oil (final concentration 1.2 mmol chlorophyll/kg diet) (Japan Chlorophyll Co., Chiyoda-ku, Tokyo) exchanged for 12 g palm fat. The control, haem and haem plus spinach diets were composed as described in Table I. An aliquot of 12 g palm oil (Japan Chlorophyll Co.) was added instead of 12 g palm fat/kg diet as a control for the chlorophyll solution.

In a third study we compared the effects of a control and haem-supplemented diet on colon histology, using a similar study design and diets as described for the first study.

Food was administered to the rats just before dark, to prevent possible degradation of the supplements. Food and demineralized drinking water were supplied *ad libitum*. Food intake and body weights were recorded every 2–4 days. Faeces were quantitatively collected during days 11–14 of the experiment and frozen at –20°C.

*In vivo colonic epithelial cell proliferation*

After 14 days of experimental feeding colon epithelial cell proliferation was quantified *in vivo* by measuring DNA replication, using [methyl-<sup>3</sup>H]thymidine incorporation into DNA. We chose this method because it is a rapid, specific and highly quantitative marker of cell replication *in vivo*. Moreover, we have shown earlier that DNA replication correlates highly with faecal water cytotoxicity (16). This substantiates a cause and effect relationship that we

want to address in the present study. Non-fasted rats were injected i.p. with [methyl-<sup>3</sup>H]thymidine (specific activity 925 GBq/mmol, dose 3.7 MBq/kg body wt; Amersham International, Amersham, UK) in 154 mM NaCl. After 2 h the rats were killed by CO<sub>2</sub> inhalation. The colon was excised and opened longitudinally. Colonic contents were removed by rinsing with 154 mM KCl. The mucosa was scraped using a spatula and homogenized (Ultraturrax Pro200; Pro Scientific Inc., Monroe, CT) in 1 ml of buffer containing 200 mM sucrose, 20 mM Tris and 1 mM dithiothreitol, pH 7.4, combined with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). These homogenates were analysed for [<sup>3</sup>H]thymidine incorporation and for total DNA content. The macromolecular fraction was precipitated with 5% trichloroacetic acid (w/v), followed by 10 min centrifugation at 10 000 g. The supernatant was discarded and the pellet resuspended in 1 M perchloric acid and hydrolysed at 70°C for 20 min. Part of the final hydrolysate (0.5 ml) was dissolved in 15 ml of Aqua Luma (Lumac-LSC BV, Groningen, The Netherlands) and its radioactivity measured in a Beckman LS-7500 liquid scintillation counter with correction for quenching. DNA content of the scrapings was determined using the diphenylamine reaction with calf thymus DNA (Sigma) as standard (17).

*Quantification of epithelial DNA in faeces*

Faecal host DNA content was quantified with real-time PCR, using a rat-specific probe and primers with the β-globin gene as target sequence (18). DNA was isolated from 20 mg freeze-dried faeces using a QIAamp DNA stool mini kit (Westburg, Leusden, The Netherlands). All isolates were of good purity (A<sub>260</sub>/A<sub>280</sub> ≈ 1.8) and were kept at 4°C or at –20°C for long-term storage. The standard DNA used for quantification of DNA in faeces was isolated from rat spleen, using a Wizard Genomic DNA purification kit (Promega Corp., Madison, WI). Real-time PCR was performed using an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Universal Taqman Master Mix and Taqman Exogenous Internal Positive Control (IPC) Reagent (Vic<sup>TM</sup> probe) were purchased from Applied Biosystems and the Taqman probe containing a FAM 5'-labelled fluorescent reporter dye (6-carboxyfluorescein) and a TAMRA 3'-labelled quencher dye (6-carboxytetramethylrhodamine) was purchased from Prologo (Paris, France). PCR primers were synthesized by the Amersham Pharmacia Biotech Custom DNA Synthesis Service (Roosendaal, The Netherlands). For amplification of host DNA, 5 μl of the purified DNA (100–200 ng) was used in a volume of 50 μl, containing 1× Master Mix, 200 nM forward primer (5'-TGATGGCCTGAAACACTTGG-3'), 200 nM reverse primer (5'-TCAGGATCCACATGCAGCTT-3'), 100 nM probe (5'-CAACCTCAAGGGCACCTTTGCTCA-3'), 1× IPC mix and 1× IPC DNA. The PCR protocol consisted of 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 62°C.

*Histological measurements*

After 14 days on the control or haem diet, the rats in the third study were killed by CO<sub>2</sub> inhalation and their colons excised for histological evaluation. A sample of the colon was stored in 10% neutral buffered formalin. The samples

were embedded in paraffin and sectioned to 4  $\mu\text{m}$  slides, which were stained with haematoxylin and eosin. The slides were examined by light microscopy (Reichert-Jung, Polyvar, Austria) and the image was captured with a colour video camera (Leica DC 200, Switzerland) coupled to a desktop computer. Calibrated software (Leica DC Image Acquisition, Leica Microsystems, Switzerland) was used to measure crypt column height. A minimum of five crypts per slide were recorded by an observer who had no knowledge of their identity. We also studied apoptosis on the paraffin embedded slides with terminal deoxynucleotidyl transferase d-UTP nick end labelling (TUNEL) (DNA Fragmentation Detection Kit, TdT-FragELTM; Oncogene Research Products).

#### Preparation of faecal water

Faecal water was prepared by reconstituting a small amount of freeze-dried faeces with double-distilled water to obtain a physiological osmolarity of 300 mOsm/l, as described earlier (9). After centrifugation, the faecal waters were stored at  $-20^{\circ}\text{C}$  until further analysis.

#### Determination of haem in faeces

Total amount of haem excreted in the faeces was determined by a previously described HemoQuant assay (19). To quantify haem in faeces an acidified chloroform-methanol extract (20) was obtained from 30 mg freeze-dried faeces (final HCl concentration 1 M). The chloroform phase of the extracted samples was dried under nitrogen and solubilized in 0.45 ml of 250 mM KOH. Subsequently, 0.45 ml of double-distilled water, 3.75 ml of 2-propanol and 0.75 ml of 1.15 M HCl was added to the samples and they were assayed for their haem content as described (19).

#### Cytotoxicity of faecal water

Cytotoxicity of faecal water was quantified by potassium release of a human erythrocyte suspension after incubation with faecal water as described previously (9) and validated earlier with human colon carcinoma-derived Caco-2 cells (21). The potassium content of the erythrocytes was measured with an ICP-AES and the cytotoxicity of faecal water was calculated and expressed as a percentage of maximal lysis.

#### Purification of the cytotoxic haem metabolite from faecal water

The cytotoxic haem metabolite (haem factor) was purified using lipid extraction and size exclusion chromatography. To a volume of 100  $\mu\text{l}$  of faecal water pooled per treatment group was added 560  $\mu\text{l}$  of water and 2.5 ml of diethyl ether (ether) and, after vigorous mixing, the mixture was centrifuged at 1500 g for 5 min. The ether phase containing chlorophyll but not haem was discarded, and the procedure was repeated twice (22). The remaining 660  $\mu\text{l}$  sample was acidified with 60  $\mu\text{l}$  of HCl (final HCl concentration 1 M) and further extracted with chloroform/methanol as described earlier (20). The lipid-containing chloroform phase was obtained after centrifugation (10 min, 1500 g) and evaporated under nitrogen. This extract was purified by size exclusion chromatography with chloroform/methanol/acetonitrile/triethylamine (55:30:10:5, v/v/v/v) as elution solvent. Dried lipid extracts of 100  $\mu\text{l}$  faecal water were solubilized in 250  $\mu\text{l}$  of this solvent. Then, 200  $\mu\text{l}$  were injected. We used a Waters 501 Pumping System (Waters Corp., Milford, MA), a Gilson 231 auto sampler (Gilson Medical Electronics Inc., Villiers le Bel, France) fitted with a 500  $\mu\text{l}$  loop, a Jordi GPC/divinylbenzene column ( $300 \times 7.8$  mm, 5  $\mu\text{m}$ , 500 Å; Jordi Associates, Bellingham, MA), a photodiode array detector (Shimadzu, Kyoto, Japan), to determine the absorption spectrum of the eluted compounds, and a fraction collector (Ultracrac II; LKB, Brommen, Sweden). Absorption at 400 nm was used to detect porphyrin structures. The data were processed and analysed using Shimadzu software. Fractions were collected at 1 min intervals between 0 and 12 min after injection and evaporated under a nitrogen stream. Cytotoxicity of these fractions was determined as described above for faecal water, after resolubilizing the samples in 50 mM NaOH and adjustment to neutral pH and 300 mOsm/l with 3-(*N*-morpholino)-propanesulfonic acid.

#### Statistical analysis

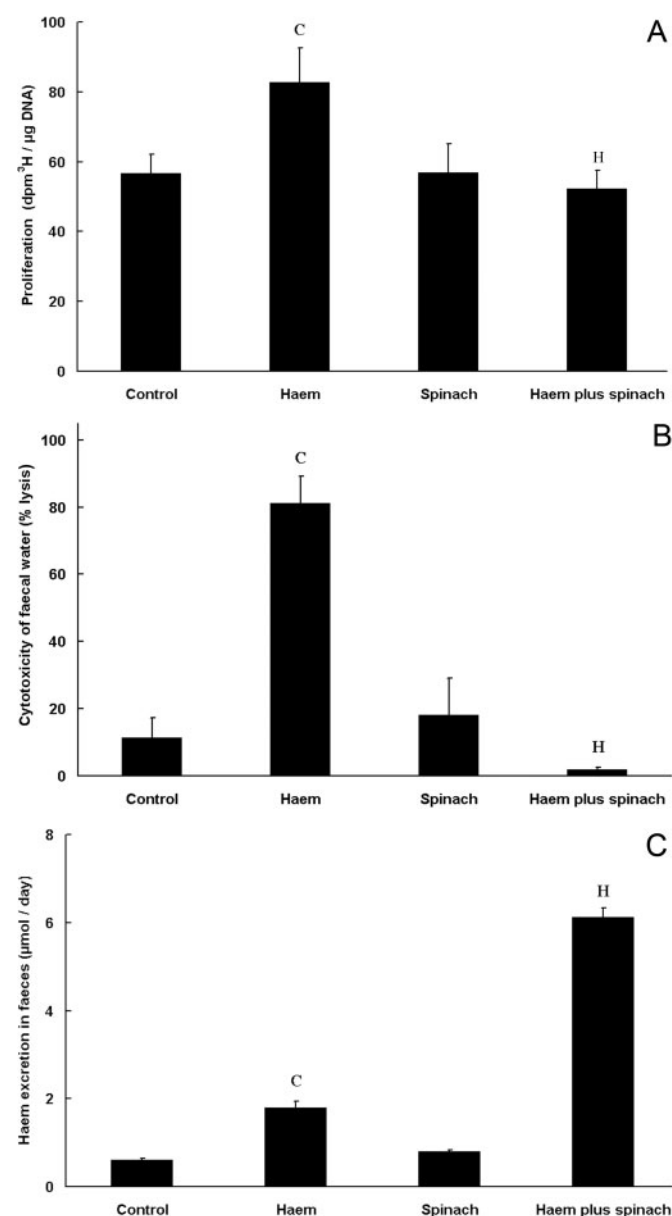
All results are expressed as means  $\pm$  SEM ( $n = 8$  per group). A commercially available package (Statistica 6.1; Statsoft Inc., Tulsa, OK) was used for all statistics. Normality of the data was tested with the Shapiro Wilk test and homogeneity of variances was tested using Levene's test. In the case of normal distribution and equal homogeneity, one-way analysis of variance (ANOVA) was performed to test for significant treatment effects, followed by Student's *t*-test. In the case of a non-Gaussian distribution of data, Kruskal-Wallis ANOVA was performed and in addition the non-parametric Mann-Whitney *U*-test was used as a *post hoc* test. Bonferroni correction was made for the number of comparisons ( $n = 3$ ). Differences were considered statistically significant at  $P < 0.05$ .

## Results

### Spinach inhibits the detrimental colonic effects of haem

First, we tested whether spinach could inhibit haem-induced colonic cytotoxicity and hyperproliferation of the epithelial cells. Rats were fed purified control and haem diets in the presence and absence of spinach. Addition of haem or spinach to the diets did not cause differences in food intake or growth rate between the groups; average food intake was  $17.0 \pm 1.2$  g/day and growth rate  $2.0 \pm 0.6$  g/day.

Proliferation of the epithelial cells was measured to determine if changes in diet resulted in different responses of the colonic epithelium. As shown in Figure 1A, the group fed haem had a 50% increase in rate of DNA replication compared



**Fig. 1.** Effect of dietary haem, spinach and haem plus spinach on: (A) colonic epithelial cell proliferation, determined by *in vivo* [methyl- $^3\text{H}$ ]thymidine incorporation into colonic mucosa; (B) cytotoxicity of faecal water, determined with an erythrocyte bioassay; (C) haem excretion in the faeces, determined by HemoQuant. Results are means  $\pm$  SEM ( $n = 8$ ). <sup>C</sup>Significantly different from control group; <sup>H</sup>significantly different from haem group ( $P < 0.05$ ).

with the control group. Supplementation of the haem diet with spinach resulted in total inhibition of this haem-induced colonic epithelial hyperproliferation. No differences in epithelial proliferation were observed between the control group and the spinach without haem group. In addition, rats fed the haem diet had  $4.3 \pm 0.15$  mg DNA/g scraping, which was significantly lower than the  $5.3 \pm 0.3$  mg DNA/g scraping of the rats fed the other diets. However, rats fed the haem diet had a significantly higher total scraping weight of  $0.54 \pm 0.04$  g than the  $0.30 \pm 0.03$  g scraping for the rats fed the other diets. This haem-specific increase in scraping weight may reflect an increased fragility and/or increased thickness of the colon mucosa. Histological observation in a third study of rats fed a control and haem-supplemented diet showed that the haem diet disrupted the structure of the surface epithelium, indicating necrosis (data not shown). In addition, haem significantly increased the crypt column height: control diet  $255 \pm 8$   $\mu$ m versus the haem diet  $324 \pm 10$   $\mu$ m. We also investigated a possible differential effect of haem versus control on apoptosis and/or necrosis more directly by end-labeling of DNA fragments in paraffin-embedded mucosa slides. In control rats we found positive staining of apoptotic bodies in a few crypt cells. In slides from haem-fed rats we always found a diffuse brown staining of the cytosol, indicating necrosis in surface cells and hardly any apoptotic crypt cells. Unfortunately, we could not further quantify these effects, because the day-to-day reproducibility of the TUNEL kit was insufficient. This requires further investigation. However, these qualitative effects at least indicate, in our opinion, that haem induces damage to surface cells and inhibition of apoptosis in crypt cells.

Subsequently we determined whether these differences in cell turnover were due to differences in exposure of the colonocytes to luminal irritants. Figure 1B shows that the cytotoxicity of faecal water of the haem group increased 7-fold compared with the control group. The addition of spinach to the haem diet abolished this cytotoxic effect of haem. Supplementation of the non-haem control diet with spinach did not affect cytotoxicity.

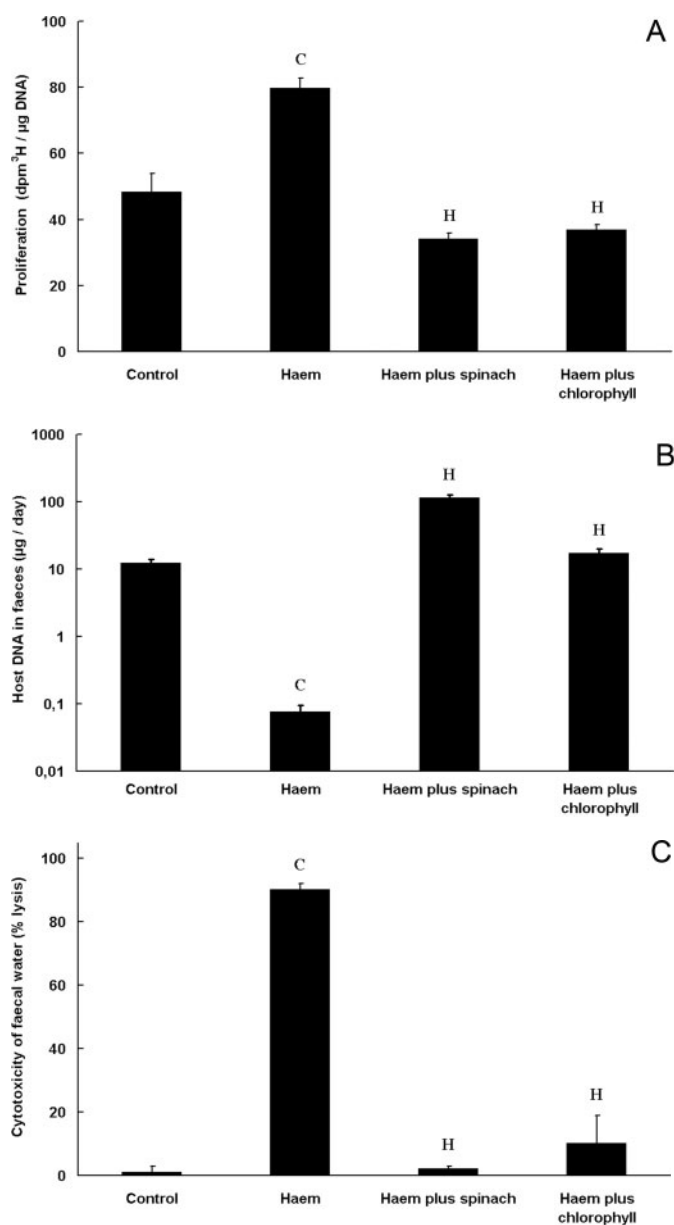
We determined the amount of native haem excreted in faeces, to study whether spinach affects intestinal haem metabolism. As expected, faecal output of haem was low on the control and the spinach diets and supplementation of the control diet with haem resulted in an increased haem excretion (Figure 1C). The amount of native haem excreted on the haem plus spinach diet was 3-fold higher than on the haem diet, even though the amounts of haem fed were equal. This indicates that spinach inhibits intestinal catabolism of haem.

These data show that spinach inhibits the haem-induced detrimental colonic effects. This raised the question which component in spinach is responsible for its protective effect.

#### *The protective effect of spinach is due to its chlorophyll content*

In a second study we investigated whether the protective effect of spinach can be mimicked by an equimolar amount of chlorophyll. Therefore, rats were fed a control, a haem and a haem plus spinach diet identical to those in the first study and a haem plus chlorophyll diet. Food intake of the different groups in the second study was  $\sim 18$  g/day. Growth rate of the rats fed haem plus chlorophyll ( $5.4 \pm 0.3$  g/day) was higher than that of the other groups ( $4.2 \pm 0.3$  g/day).

Figure 2A shows that both chlorophyll and spinach completely prevented haem-induced epithelial hyperproliferation.



**Fig. 2.** Effect of dietary haem, haem plus spinach and haem plus chlorophyll on: (A) colonic epithelial cell proliferation, determined by *in vivo* [methyl-<sup>3</sup>H]thymidine incorporation into colonic mucosa; (B) the level of host DNA detected in faeces, quantified by real-time PCR; (C) cytotoxicity of faecal water, determined with an erythrocyte bioassay. Results are means  $\pm$  SEM ( $n = 8$ ). <sup>C</sup>Significantly different from control group; <sup>H</sup>significantly different from haem group ( $P < 0.05$ ).

The effect of the different diets on proliferation of colonic epithelial cells was reproducible between studies: the effects observed for the control, haem and haem plus spinach diets were the same in study 1 as in study 2.

Subsequently, to study alterations in the fate of colonocytes, we determined whether these diet-induced differences in cell turnover resulted in differences in exfoliation of colonocytes into the faecal stream. Figure 2B shows that dietary haem strongly reduced the amount of host DNA in the faeces, compared with the control diet. Spinach and chlorophyll supplements in the haem diet abolished the haem-induced decrease in rat DNA excretion in faeces. Similar results for the haem and haem plus spinach groups were observed in the first study (data not shown).

Analogous to the results observed in the first study, supplementation of haem increased cytotoxicity of the faecal water extract compared with the control diet. Supplementation of the haem diet with chlorophyll and spinach completely inhibited this haem-induced increase in cytotoxicity (Figure 2C).

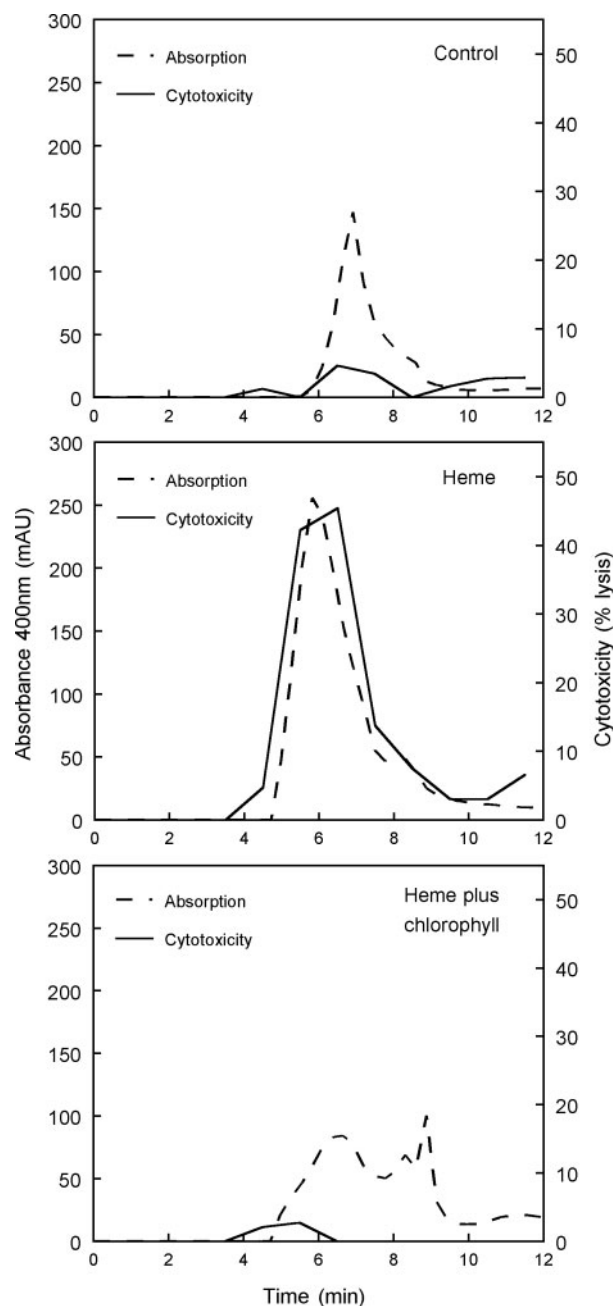
We showed earlier that a cytotoxic haem metabolite (haem factor) can be isolated by size exclusion chromatography, where its absorption at 400 nm coincides with cytotoxicity (23). We investigated the effect of chlorophyll on the formation of this haem factor. Figure 3 shows the elution profiles for UV absorption (at 400 nm) and cytotoxicity of lipid extracts of pooled faecal water of the control, haem and haem plus

chlorophyll groups. Only the faecal water extract of the haem group contained UV-absorbing cytotoxic components eluting around 6 min. The UV absorption spectra of these fractions showed that they contained the porphyrin structure of haem. Because native haem elutes later than 12 min (23), this implies that cytotoxicity is due to a haem metabolite of higher molecular weight. It should be noted that cytotoxicity of the pooled fractions was always >90%, indicating that there was no loss of haem factor during extraction and chromatography. Unfortunately, we could not identify the molecular structure of the covalently bound haem metabolite because it resisted ionization in different types of mass spectrometers. Nevertheless, Figure 3 clearly shows that dietary chlorophyll blocks formation of the haem factor almost completely. A similar inhibition was found for the haem plus spinach group (data not shown).

## Discussion

This study shows for the first time that spinach and chlorophyll inhibit haem-induced stimulation of colonic epithelial cell turnover. In line with our previous rat studies, dietary haem induced colonic cytotoxicity and compensatory hyperproliferation of colonic epithelial cells (9), which may increase endogenous mutations and thus colon cancer risk (12,24). This possible carcinogenic effect of haem has also been studied by Pierre *et al.* (25), who showed that dietary haem promotes luminal cytotoxicity and growth of aberrant crypt foci in rat colon to the same extent. These results imply that haem-induced cytotoxicity disturbs normal cell turnover in colonic epithelium. Colonocytes differentiate and mature during their migration along the crypt to the surface. At the end of their life-cycle these surface cells become senescent and are engulfed by stromal cells or exfoliated into the faecal stream (26,27). Our preliminary histological data indicate that haem damages surface colonocytes, which could explain the decreased exfoliation of senescent surface cells. The compensatory colonic hyperproliferation in the haem-fed rats implies that more surface cells are killed than are exfoliated in control rats. At present we do not know why haem also increases crypt column height and cell number (DNA content) in the mucosa. Whether this is due to inhibition of apoptosis in crypt cells requires more detailed immunohistochemical and cell kinetic studies. Nevertheless, the effects of haem on luminal cytotoxicity, colonocyte proliferation and mucosal cell number were prevented by supplementing the haem diet with spinach. Moreover, this protective effect of spinach could be mimicked completely by an equimolar amount of chlorophyll, indicating that other components in spinach, such as fibre, are not responsible.

Consistent with our previous work, we have shown that haem-induced cytotoxicity is due to the presence of an unknown lipid-soluble cytotoxic haem metabolite (haem factor) (23). The haem factor is a covalently modified porphyrin, formed from haem in the gastrointestinal tract of the rat (9,23). We were unable to ionize this haem factor, so no mass spectrum could be obtained. Similar difficulties in identifying the structure of haem metabolites have been reported by others (28–30). Spinach increased haem recovery in the faeces with equal intake of haem. This could indicate that spinach protects haem and prevents it from degradation. Purification of the haem factor in the second study showed that chlorophyll not



**Fig. 3.** Representative size exclusion chromatograms of lipid extracts of pooled faecal waters from rats fed a control, haem or haem plus chlorophyll diet, measured by the absorption at 400 nm (dashed lines). One minute fractions were collected from 0 to 12 min and assayed for cytotoxicity using an erythrocyte bioassay (continuous lines).

only prevented haem degradation, but also inhibited formation of haem factor, concordant with the low cytotoxicity of the colonic contents.

How does chlorophyll inhibit haem-induced colonic cytotoxicity and subsequent adverse effects? The addition of chlorophyll to a haem diet prevented formation of haem factor. Because the molecular structure of the haem factor is still enigmatic, we can only speculate how spinach and chlorophyll inhibit its formation. Two possible inhibitory mechanisms can be proposed: competition in the solubilization process of haem and blocking of the modification sites of haem. Haem is poorly soluble in the stomach due to the low gastric pH (31). However, in order to form haem factor, haem must be solubilized. This solubilization is accomplished in the proximal small intestine by bile acids and other surfactants such as fatty acids and is probably the first important step in the formation of haem factor (32). Chlorophyll might prevent haem solubilization by competition for binding to bile acids and other surfactants in the proximal small intestine. Alternatively, chlorophyll could 'sandwich' haem to form hydrophobic haem-chlorophyll complexes and, as a result, block the covalent modification sites of haem and thus the formation of haem factor.

Most epidemiological studies show that red meat promotes and vegetable consumption decreases the risk for colon cancer (4,5,14). The mechanism underlying modulation of colon cancer risk by dietary intake of red meat and vegetables is still unknown. Butler *et al.* (33) suggest an association between colon cancer risk and heterocyclic aromatic amines formed during cooking of meat. However, this is not supported by other epidemiological evidence (34). In addition, Sinha *et al.* (35) showed that the level of some heterocyclic aromatic amines in cooked white meat exceeds the level in cooked red meat, which implies that heterocyclic amines cannot explain why red meat raises the risk while white meat does not. Besides, doses of heterocyclic aromatic amines required for carcinogenicity in animal studies far exceed the daily dietary intake by humans (10). Bingham *et al.* (36,37) proposed another mechanism, suggesting that haem-induced formation of *N*-nitroso compounds from red meat is responsible for the increased colon cancer risk. However, green vegetables did not decrease the concentration of apparent *N*-nitroso compounds in a follow-up study from the same group (38). This could be due to the type of vegetables consumed and/or the small number of participants in the study and indicates that further studies on the modulation of colon cancer risk by *N*-nitroso compounds and components from green vegetables are needed.

Because our experimental diets mimicked the macronutrient composition of a low calcium, high meat and high fat diet our results may have implications for the human situation. We showed previously that dietary haem concentrations between 0.16 and 0.5 mmol/kg diet resulted in similar increases in cytotoxicity of the colonic contents and proliferation of the epithelial cells (23). Assuming that an average human diet consists of ~450 g dry weight/day, 0.16 mmol/kg haem corresponds to 72 µmol haem/day. As beef contains up to 0.5 µmol haem/g wet weight (29), this implies a realistic beef intake of 150 g/day in humans.

In our rat studies a concentration of 1.2 mmol/kg chlorophyll inhibited the haem-induced cytotoxicity. Considering that spinach contains 0.12 mmol chlorophyll/100 g wet weight (39), people consuming 450 g of spinach/day could be protected

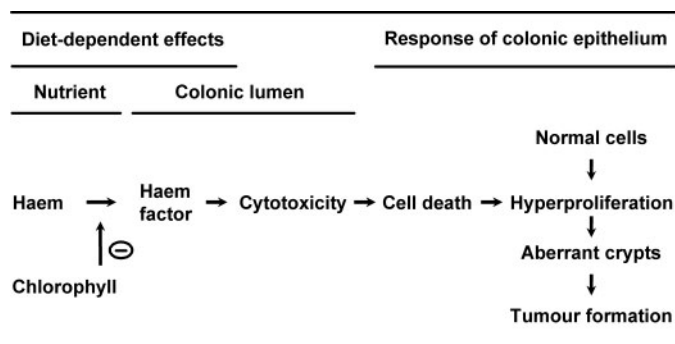


Fig. 4. Proposed mechanism for the interaction between dietary haem and chlorophyll in the lumen of the colon and effects on the colonic epithelium.

from the adverse effects of dietary haem on the colonic mucosa. This protective effect of spinach is a maximum estimation and will be smaller if the effective inhibiting chlorophyll concentration is lower than the 1.2 mmol/kg used in the present study. The concentration dependence of this protective effect of chlorophyll is at present under investigation.

Figure 4 summarizes our studies on haem and chlorophyll, showing that modulation of colon cancer risk is a result of antagonistic interactions in the gut lumen. Our proposed mechanism implies that people who consume a diet high in red meat and low in green vegetables are especially at risk for colon cancer. We suggest that this haem-chlorophyll interaction should be taken into consideration in future epidemiological and experimental studies.

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