Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons.

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Beef meat and blood sausage promote azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons[1]

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Running title: Red meat promotes colon carcinogenesis in rats

Foot notes:

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3- Abbreviations: ACF: aberrant crypt foci, MDF: mucin-depleted foci, MTT: 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromid, TBARS: thiobarbituric acid reactive substances

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Abstract

Red meat is associated with colon cancer risk. Puzzlingly, meat does not promote carcinogenesis in rodent studies. However, we demonstrated previously that dietary heme promotes aberrant crypt foci (ACF), in rats given a low-calcium diet. Here, we test the hypothesis that heme-rich meats promote colon carcinogenesis in rats treated with azoxymethane, in low-calcium diets (20 mmol/kg). Three meat diets were formulated to contain varying concentrations of heme by the addition of raw chicken, beef, or black pudding (blood sausage). The no-heme control diet was supplemented with ferric citrate, and a heme control diet with hemoglobin, to match iron or heme concentration in the beef diet. After 100 d colons were scored for ACF and mucin-depleted foci (MDF). Fecal water was assayed for lipoperoxides and cytotoxicity. Only diets with heme promoted MDF, but all meat diets promoted ACF. The number of MDF/colon was 0.55±0.68 in controls, but 1.2±0.6 ($p=0.13$), 1.9±1.4 ($p<0.01$) and 3.0±1.2 ($p<0.001$) in chicken, beef and black pudding-fed rats. MDF promotion was significantly greater for the high-heme black pudding diet, than for the median-heme beef diet. The number of ACF/colon was 71±16 in controls, but 90±18, 99±13 and 103±14 in chicken, beef, and black pudding-fed rats (all $p<0.001$). No ACF or MDF difference was seen between beef and the matching heme control diet. MDF promotion correlated with high fecal water lipoperoxides and cytotoxicity ($r=0.65$, $p<0.01$). This is the first study to show the promotion of experimental carcinogenesis by dietary meat, and the association with heme intake.

Keywords: Colorectal carcinogenesis, Heme, Lipoperoxidation, Red meat, Chicken

Introduction

Colorectal cancer is a major killer in affluent countries, and recommendations are to reduce red meat intake to reduce the risk (1). The meta-analysis of epidemiological studies by Norat *et al.,* found a moderate but significant association between red meat intake and colorectal cancer risk (2). In puzzling contrast with epidemiological studies, experimental studies do not support the hypothesis that red meat would increase colorectal cancer risk. Among the twelve rodent studies reported in the literature, none demonstrated a specific promotional effect of red meat (3-14). McIntosh *et al.* (3) showed that rats given a diet containing kangaroo meat, soybean protein or casein have similar incidence of dimethylhydrazine-induced tumors. Clinton *et al.* (4) also found the colon tumor incidence to be the same for beef meat (raw or grilled) and soybean diet fed rats. Nutter *et al.* (5) found beef proteins to afford significant protection in mice compared with milk protein. Reddy *et al.* (6) and Pence *et al.* (7) found high-protein and high-fat diets, whatever the protein source, to increase colon tumor incidence in rats, but beef meat affords a significant protection compared with casein (7). Pence *et al.* (8) found that well-cooked beef meat decreases the risk of colon cancer in rats compared to casein in a high-fat context, but increases the risk in a low-fat context. Lai *et al.* (9) found that a lean beef diet does not increase tumor incidence in rats compared with a casein-iron citrate diet. Alink *et al.* (10) showed that human diets (with meat) produced more colon carcinomas in rats than rodent diets (with no meat). Alink's results do not support specific meat promotion, however, as the human diets contained more fat and less fiber than the rodent diets. Mutanen *et al.* (11) did not find beef meat diet to increase significantly the number of intestinal tumor in Min mice, although it contained five times more fat than the control diet. Ketunen et al. (12) found less tumors in female Min mice given beef meat than in controls. Parnaud *et al.* (13) did not find red meat to promote azoxymethane-induced aberrant crypt foci (ACF) compared to casein-fed controls. Belobrajdic *et al.* (14) found

kangaroo meat to promote ACF in comparison with whey protein, but whey is a known protector of colon carcinogenesis (15).

Sesink *et al.* speculated that heme, found in red meat myoglobin, would enhance colon carcinogenesis. They demonstrated that pure hemin added to rats diet increases colonic epithelial proliferation (16), and that calcium phosphate inhibits the hemin-induced proliferation (17). In line with Sesink's hypothesis, we have shown that hemin diets increase the number and size of azoxymethane-induced ACF in rats fed a low-calcium diet, while hemoglobin diets increase ACF number only (18). Dietary hemin also produces cytotoxic fecal water and high amounts of thio-barbituric acid reactive substances (TBARS), indicative of lumen lipoperoxidation (16), while dietary hemoglobin increases fecal TBARS only (18). ACF are putative preneoplastic lesions, and the effect of agents on ACF correlates with the effect on tumor incidence in most studies (19), but not all. Recently, alternative short term biomarkers of colon carcinogenesis have been proposed: mucin-depleted foci (MDF)(20). MDF are easy to score and may predict tumor outcome better than ACF (20,21).

The present study was designed to test the hypothesis that heme in the food matrix can promote colon carcinogenesis in a low-calcium context. The diets used in previous animal studies (3-13) contained high levels of calcium, which may explain they did not show a promoting effect of red meat. Three types of meat were chosen with different heme contents: Chicken, beef and black pudding. A fourth diet, containing pure hemoglobin, was added. This diet acted as a control as it contained the same concentrations of heme as the beef diet, and the myoglobin in beef is very close in structure to hemoglobin. Besides the ACF endpoint, we also scored MDF.

Materials and methods

Animals

Sixty Fischer 344 female rats were purchased at 4 w of age from Iffa Credo (St.Germain l'Arbresle, France). Animal care was in accordance with the guidelines of the European Council on animals used in experimental studies. They were distributed randomly in pairs into stainless steel wire bottomed cages. The room was kept at a temperature of 22°C on a 12-h light-dark cycle. Animals were allowed 7 d of acclimatization to the room and to the control diet (cf. Table I) before being injected i.p. with the carcinogen azoxymethane (Sigma chemical, St.Quentin, France; 20 mg/Kg body weight) in NaCl (9 g/L). Seven days after the injection the rats were allowed free access to their respective diet for 100 d. Feed was changed every second or third day and water once a week. Animal body weights were monitored weekly. Feed intake per cage of two rats was also monitored at periodic intervals (d 5, 62 and 77). Fecal mass was measured as the total over a 24 h period per two rats on d 56, 61, 62, 76 and 77.

Diets

Experimental diets, as shown in Table I, were based on the diet fed to control rats (N=20 rats) consisting of a modified AIN-76 diet (22) prepared and formulated in a powdered form by the UPAE (INRA, Jouy-en-Josas, France). Dibasic calcium phosphate was included at a low concentration of 20 mmol/kg. Three meat diets given to three groups of rats (N=10 rats/group), were formulated to contain varying concentrations of heme as hemoglobin or myoglobin by the addition of freeze-dried beef, chicken or black pudding at 60% (w/w) meat of the total diet by weight. The beef and chicken (skin-less) meat was obtained from UPAE. Meat was freeze-dried by LyoFal (Salon de Provence, France). The beef contained 0.6 μ mol/g of heme while none was detected in the chicken diet (see the assay below). The low fat

black pudding (blood sausage) contained 16 µmol/g of heme. It was specially made by Recape (Lanta, France) with 90% pork blood and 10% starch (w/w), and contained no potentially protective additives such as onion or milk. One group of rats (N=10) received a hemoglobin diet containing the same concentration of heme as the beef diet (0.36 µmol/g diet). This was achieved by adding powdered bovine hemoglobin (Sigma chemical, St.Quentin, France) to the control diet. All diets were balanced for protein (50%), fat (20%), calcium (20 mmol/kg) and iron (2.5 mmol/kg) by addition of casein, lard, calcium phosphate and ferric citrate. However, the black pudding died could not be balanced for iron (17 mmol/kg). The diets were made up twice a month and maintained at –20°C, and TBARS assay showed no lipoperoxidation (data not shown).

ACF and MDF Assays

All rats were killed by CO2 asphyxiation in a random order on day 99 or 100. Coded colons were scored for ACF by Bird's procedure (23). ACF scoring was done in duplicate by two readers not knowing the rat treatment. Colons, after being scored for ACF, were stained with high iron diamine-Alcian blue procedure (HID-AB) to evaluate mucin production (20). MDF number, and number of crypts per MDF, were scored by a single reader, not knowing the rat treatment or the ACF results, under light microscope at x32 magnification. According to Caderni *et al.* , lesions were identified as MDF by the absence or very small production of mucins, and by at least two of the following criteria: multiplicity higher than 3 crypts, distortion of the lumen of the crypts, elevation of the lesion in comparison of normal mucosa (20). All lesions were photographed (Figure 1), and representative pictures were submitted by mail to Dr. Caderni for confirmation.

Preparation of Fecal Water, Assay of TBARS and Heme.

For assay of TBARS, heme, and cytotoxic activity on CMT93, fecal water was prepared from 24-h feces collected under each cage of two rats, as previously described (18), but black pudding samples were diluted twice more than other samples. For assay of cytolytic activity on erythrocytes, fecal water was prepared by Sesink's procedure and pH measured (16). TBARS were measured in fecal water according to Ohkawa *et al.* (24), exactly as previously described (18). Heme contents of freeze-dried feces and of fecal water were measured by fluorescence according to Van den Berg *et al.* (25) and to Sesink *et al.* (16), respectively, as already described (18).

Cytolytic Assay of Fecal Water

The cytotoxicity of fecal water was quantified by two methods, on erythrocytes, and on a cell line. First, the cytolytic activity of fecal water was quantified by potassium-release from erythrocytes as described by Govers *et al.* (26). Secondly, the cytotoxicity of fecal water obtained with a different method (see above) was also quantified by the MTT test on a cell line according to Bonneson *et al.* (27). Briefly, the cancerous mouse colonic epithelial cell line, CMT93 (ECAC), was seeded in 96-well microtiter plates (1.6×10^4 cells per well in 200 µL of medium) and at confluence the cells were treated for 24 h with the fecal water sample to be tested diluted in the culture medium at a concentration of 0.1% (v/v). Each fecal water sample was tested in 7 wells and 10 wells remained untreated to act as controls. One hundred µL of MTT (9% in PBS) was added to each well. After 3 h of incubation at 37°C in the dark, 100 µL of a 10% SDS - 0.1 mol/L NaOH mixture was added. After 1 h of incubation in the dark, the absorbance of each well was read using a microplate reader at wavelength 570 nm for cytotoxicity and 690 nm for background.

Statistical Analysis

Results were analyzed using Systat 10 software for Windows, and reported as mean ± SD. ACF scoring was done in duplicate. Values of ACF were considered firstly using two-way (groups and readers) analysis of variance (ANOVA). The (group x reader) interaction was never significant, and when total ANOVA was significant ($p<0.05$), pairwise differences between groups were analysed using the Fishers's least-significant-difference test. MDF values and all other data were considered using one-way ANOVA and groups were compared using the Fishers's least-significant-difference test. The Pearson coefficient was used to determine the correlation between ACF, MDF, heme intake and fecal values, and p values were calculated with Bonferronni correction for multiple comparisons. Because the black pudding diet contained a very high concentration of heme, heme values were log-transformed before statistical analysis.

Results

Weight gain and feed intake

Beef-fed rats quickly became heavier than control rats, and the difference reached significance at d 30. Final body weight of beef-fed rats was 210±9 g (cf. Table 2), higher than controls 198±12 g ($p<0.05$). Black pudding-fed rats had watery stools, a known effect of dietary heme, and they drank more water than controls (22±1 ml/d vs 16±0.5 ml/d, $p<0.001$). Furthermore, all groups had similar food intake, the mean value being 8.4±0.5 g/d at day 75 (full data not shown).

ACF data

All meat-based diets (chicken, beef and black pudding) significantly increased the number of ACF ($p<0.001$, Figure 2A) and the number of aberrant crypts per colon ($p<0.001$, Table 2) after 100 d on the diets. Chicken and black pudding, but not beef, also increased the number of crypts per ACF ($p<0.01$, Table 2). Aberrant crypts and ACF promotion by black pudding diet was significantly more potent than promotion by chicken diet ($p<0.05$, Table 2). No significant difference was seen between rats given the beef diet and rats given the matching hemoglobin diet for aberrant crypts or ACF per colon, but the size of ACF was significant greater for haemoglobin group (Table 2).

MDF Data

Beef and black pudding-fed rats had more MDF than control rats ($p<0.01$), and promotion by black pudding was more potent than promotion by beef ($p<0.05$, Figure 2B). Chicken-based diet, which contains no heme, did not promote MDF (Figure 2B). The effects observed on the number of MDF were also observed on the number of mucin depleted crypts (Table 2). No

differences were observed between groups in the number of crypts per MDF. Last, no significant difference was observed between the beef and hemoglobin groups (table 2).

Fecal heme, TBARS and Cytotoxicity

The heme intake, and the fecal concentration of heme, matched the study design. As expected, no heme was detected in feces of control and chicken diet fed rats (Table 3). The analysis of fecal samples stored during previous meat study of the laboratory where diet containing too 60% beef meat but 130 µmol/g calcium (13) yielded similar results: No heme was detected in feces of control and chicken diet fed rats, but 1.7±1.5 µmol/g in feces of beef meat fed rats. However, in the present study, the heme concentration was higher in the feces of hemoglobin-fed rats than in beef-fed rats (Table 3). This fits with the observation that less heme iron reaches the colon when it is supplied as red meat rather than in hemoglobin form (14). We measured the characteristics of fecal water because, according to studies on bile acids, the soluble fraction of colonic contents would interact more strongly with the mucosa than the insoluble fraction (28). As expected, the heme concentration in fecal water depended directly on the level of heme in the diet (Table 3), with, as noted above, a difference between meat and hemoglobin-fed rats. No heme was found in fecal waters stored during Parnaud's meat study, even in samples from rats given a 60% beef meat diet (13).

Heme can induce the formation of peroxyl radicals in fats, which may be cytotoxic and cleave DNA *in vivo* (29). Lipid peroxidation was thus measured in fecal water by the TBARS assay. Lipid peroxidation was associated with heme concentration in fecal water (Table 3): The black pudding diet thus increased TBARS in the fecal water by 23-fold. The hemoglobin diet and beef diet increased TBARS by 2-4 fold (all $p<0.01$), but chicken diet did not change significantly fecal water TBARS, compared with control diet.

Furthermore, the fecal water of hemin-fed rats is cytotoxic, which would explain the hemin-induced increased proliferation (18). Cytotoxicity of fecal water was thus measured by two methods: lysis of erythrocytes, and toxicity on CMT93 cell in culture. The black pudding diet, a very high source of heme, enhanced erythrocytes cytolysis by more than 50-fold, and toxicity on CMT93 cells by 8-fold (both $p<0.001$, Table 3). Beef and hemoglobin diets produced equivalent effects: no lytic activity on erythrocytes, but a four-fold increase in CMT93 cells toxicity ($p<0.001$). The cytotoxicity of fecal water from chicken-fed rats was not different from that of controls (Table 3). pH value of fecal waters was also measured. All meat-based diets increased the pH value, and fecal pH was higher when heme concentration was higher in the diet (Table 3). Taken together, these data suggest that, cytotoxicity, pH and lipoperoxides of faecal water follow heme intake and fecal heme. Indeed, significant correlations were seen between heme intake and fecal water cytotoxicity ($r=0.98$), pH ($r=0.86$) and TBARS ($r= 0.73$, all $p<0.01$, $N=30$ cages).

Discussion

This study is the first to show that meat can specifically promote colon carcinogenesis. In addition, the promoting effect was strong compared with other promoting agents (30), and clearly associated to heme concentration in meat. This study was done with a low calcium diet containing 60% meat and 5% easily oxidized oil. We used two putative pre-cancerous endpoints: the established ACF, and the recently described MDF. Heme in the diet led to ACF and MDF promotion in the colon. The no-heme chicken-based diet did not promote MDF, but increased the ACF number.

This study is, to our knowledge, the first non-Italian study to use a new carcinogenesis endpoint which was recently discovered by Caderni *et al.* (20). MDF may predict tumor outcome better than ACF, as shown in the studies of synbiotics, cholic acid and piroxicam (20,21). We found that MDF were quite easy to score, but we detected fewer MDF per control rat than did Caderni. This is likely the result of the carcinogen dose: azoxymethane was injected once instead of twice, and the resulting number of ACF was four times fewer here than in Caderni's study (72 vs. 298 ACF/colon).

That heme content in meat was responsible for promotion, at least in part, is supported by the following facts: (i) All tested meat diets promoted ACF formation, but this was significantly greater for the high heme diet, based on black pudding, than for the no-heme chicken diet (Figure 2). (ii) Only heme-containing diets promoted MDF formation, and the effect was dose dependent, since black pudding effect was significantly stronger than beef effect. MDF per colon correlated with heme intake ($r = 0.63$, $N=60$, $p<0.01$). (iii) Beef and hemoglobin diets, which provided identical heme intake, promoted ACF and MDF equally (Table 3). This

meat study is thus consistent with our previous study, where ACF were promoted dose-dependently by graded doses of dietary hemin (18). We think that previous rodents studies failed to show that red meat promotes carcinogenesis, because meat was included in a high-calcium diet. The standard AIN-76 diet contains 130 mmol/kg calcium, which is similar to the concentrations that inhibits heme-induced colonic proliferation (17) and heme-induced ACF promotion (18). Calcium precipitates heme in the gut lumen, and reduces heme concentration in fecal water (17,18). In Parnaud's study (13), the heme concentration was high in the feces of beef-fed rats, but not detectable in the fecal water (see results above). We suggest this be due to high dietary calcium, and it resulted in the lack of ACF promotion by beef-meat diet (13). However, the link between heme intake and ACF yield is not a direct one: black pudding provided a huge quantity of heme to the gut that was not mirrored linearly in the ACF outcome.

The mechanism of heme promotion is not known, but might be linked to peroxidation, cytotoxicity and pH. In a previous study, we showed that pure hemin and hemoglobin promote ACF formation, and strikingly induce lipoperoxidation and cytotoxicity of fecal water (18). Indeed, heme promotes the non-enzymatic peroxidation of polyunsaturated fatty acids (16,18,29). The lipid peroxyl radicals (LOO•) generated from simultaneous fat and heme iron ingestion, and the resulting oxygen radicals, can cleave DNA or modify DNA bases, which could increase carcinogenesis (29). The beef-based diet contained 0.36 µmol/g heme. Its intake led to 19 µmol/L heme in fecal water, and a 2.5-fold increase in lipoperoxidation (Table 3). Similar TBARS values were seen in fecal water from beef-fed rats and, in our previous study (18), from hemoglobin-diet fed rats (138 and 187 mol/L MDA equivalents, respectively). In addition, red meat intake induced fecal cytotoxicity and increased pH value of fecal water (Table 3). Black-pudding contains 25 times more heme

than beef meat. Compared with beef, its consumption led to 60 times more heme in fecal water, seven times more TBARS, and a much higher cytotoxicity (Table 3). Fecal water from beef-fed rats or hemoglobin fed rats (18) did not induce cytolysis of erythrocytes, probably because heme intake was too low. In contrast, fecal water from black-pudding-fed rats strikingly induced erythrocyte cytolysis. Thus, it can be concluded that there was a dose-dependent effect of the heme concentration in the diet and in fecal water on the fecal lipoperoxidation, cytotoxicity and pH, and these values correlated significantly. In addition, MDF and ACF numbers per rat also correlated with these fecal values (all $r>0.5$, all $p<0.01$, N=60 rats, highest correlation, r=0.65 between number of MDF and cytotoxicity). These correlations suggest that fecal cytotoxicity, lipoperoxides and pH may explain heme promotion. That hemoglobin and meat diets, with same heme content as hemoglobin and myoglobin, produced the same effects also supports this idea (Table 3). Surprisingly, no ACF promotion was seen in a published study with a protocol very similar to this one (14). Fecal heme concentrations were similar in both studies, but fecal TBARS value was twice higher in this study. We suppose that lipoperoxidation was inhibited by *tert*-Butylhydroquinone in the AIN-93 diet used by Belobradjic (14). This chance observation supports the implication of heme-induced lipoperoxidation in heme-induced colon carcinogenesis.

The no-heme chicken-based diet surprisingly increased the ACF number and size (Table 2). ACF promotion was small yet significant, and MDF increase was not significant, which reduces the level of evidence that chicken contains a promoter. ACF data however suggest that the chicken meat used in this study may contain a promoter that is not heme, and remains to be explained. The prominent features of chicken diet were high arachidonic acid and niacin. Chicken diet contained 1 g/kg of arachidonic acid (calculated from ref.(31)) compared to 0.25 g/kg in other diets. Arachidonic acid has pro-tumorigenic properties, likely by

increasing prostaglandin synthesis (32). In addition, chicken diet contained 207 mg/kg of niacin, four times the 51 mg/kg found in control diet and twice the value in beef diet (assays done by LARA lab., Toulouse, France). Niacin can afford protection against carcinogenesis when added to a niacin-deficient diet (33), but high doses are toxic. Here, the high dose brought by the chicken-based diet would translate to 12 times the Recommended Dietary Allowances in humans (USA National Academy of Sciences). High niacin stimulates histamine release and prostaglandin synthesis, which might explain the ACF promotion (34). The intake of white meat is not associated with colorectal cancer risk in most epidemiological studies (1,2). In contrast, dietary heme iron intake is associated with an increased risk of proximal colon cancer (35). However, in a prospective cohort study of 34198 Californian Adventists, the consumption of white meat, mostly chicken, was associated with a tripled risk or colorectal cancer (36).

In summary, this study shows for the first time a promoting effect of red meat on carcinogenesis. It corroborates epidemiological observations: high red meat intake is associated with increased colon cancer risk. In previous meat studies (3-13), the promoting effect of meat was hidden by dietary calcium, as shown here on Parnaud's study (13). Furthermore, MDF promotion was related to heme intake. Promotion was significantly greater for the high heme black pudding diet, than for the median-heme beef diet. This heme effect matches latest epidemiological data (35). The low-heme chicken diet did not promote MDF, but did increase ACF formation. For red meat diets, promotion was associated with high fecal water lipoperoxidation, cytolytic activity and increase of pH, which may explain the increased carcinogenesis.

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Table I: Composition of diets (g/kg)

	Control	Chicken	Beef	Black Pudding	Hemoglobin
Chicken	0	600	0	0	0
Beef	0	0	600	0	0
Black Pudding	0	0	0	600	0
Hemoglobin	0	0	0	0	6.3
Lard	150	122	40	112	150
Safflower Oil	50	50	50	50	50
Casein [a]	500	1.1	48.5	115	493.8
Corn Starch	60	60	60	5	60
Sucrose	139.5	68	102	20	139.5
Cellulose	50	50	50	50	50
Methionine	3	3	3	3	3
Mineral mix [b]	35	35	35	35	35
Vitamin mix [b]	10	10	10	10	10
$CaHPO_4.2H_2O$	2.7	1.4	1.6	1.8	2.7
Ferric Citrate [c]	0.45	0.35	0	0	0.36

a. Low-calcium casein

b. AIN76 mix, but 500g/kg of dibasic calcium phosphate replaced by sucrose in mineral mix.

c. All diets contained 2.5 mmol/kg iron except the black pudding diet (17 mmol/kg). Iron concentration was measured in freeze-dried meat before preparing the diets: chicken: 37.5, beef: 172.6, and black pudding 1527 mg/kg. Other nutrients were balanced: 50% protein, 20% fat, 18-25% carbohydrate, and 20 mmol/kg calcium (based on added components, no analysis was done on whole diets).

Table 2: Effect of meat-based diets on aberrant crypt foci and mucin-depleted foci in the colon of rats 107 d after the injection of azoxymethane[1].

	Heme	Number	Final body	ACF			MDF		
Diets	µmol/g diet	of rats	Weight (g)	ACF/colon	ACF crypts/colon	Crypts/ACF	MDF/colon	MDF crypts/colon	Crypts/MDF
Control	0.0	20	198±12 [a]	72±16 [a]	192±55 [a]	2.7±0.4 [a]	0.55±0.68 [a]	2.9±4.0 [a]	4.65±2.40 [a]
Chicken	0.0	10	199±10 [a]	91±18 [b]	267±65 [b]	2.9±0.4 [b]	1.20±0.63 [a]	6.0±3.9 [a,b]	4.92±1.64 [a]
Beef	0.36	10	210± 9 [b]	100±13 [b,c]	280±49 [b]	2.8±0.2 [a]	1.90±1.37 [b]	8.5±6.9 [b,c]	4.23±1.15 [a]
Hemoglobin	0.36	10	196±11 [a]	93±24 [b,c]	285±78 [b]	3.1±0.5 [b]	2.40±1.50 [b,c]	11.5±9.0 [c,d]	4.60±1.93 [a]
Black Pudding	9.54	10	189± 9 [a]	103±14 [c,d]	301±48 [b]	2.9±0.2 [b]	3.00±1.24 [c]	13.1±6.0 [c,d]	4.29±0.59 [a]

1 Values are means ± SD. N= 10 rats/group, except 20 controls.

Means without a common letter differ, $P<0.05$

Table 3: Effect of meat-based diets on fecal values in rats, notably heme, lipoperoxides and cytotoxicity of fecal water[1].

Diet	Heme intake [2] µmol/d	Dry fecal Mass g/d	Heme in feces [2] µmol/g	Heme in fecal water [2] µmol/L	TBARS in fecal water MDA equivalents µmol/L	pH of fecal water pH	Cytolytic activity on erythrocytes % K release	Cytotoxicity on CMT93 cells % cells lysed
Control	0 [a]	0.50±0.11 [a]	0 [a]	0 [a]	40±15 [a]	7.85±0.03 [a]	1±2 [a]	12±12 [a]
Chicken	0 [a]	0.58±0.06 [b,c]	0 [a]	0 [a]	69±16 [a]	8.02±0.03 [b]	1±2 [a]	26±15 [a]
Beef	3.0±0.4 [b]	0.64±0.09 [b,c]	0.5±0.2 [b]	19±7 [b]	138±17 [b]	8.17±0.03 [c]	1±2 [a]	59±14 [b]
Hemoglobin	2.9±0.4 [b]	0.53±0.07 [b]	0.9±0.3 [c]	52±47 [c]	195±96 [b]	8.13 [c]	1±1	58±27 [b]
Black pudding	87.0±8.0 [c]	1.00±0.06 [d]	23.6±8.6 [d]	1097±484 [d]	975±229 [c]	8.30±0.06 [d]	73±36 [b]	88±03 [c]

1 Values are means ± SD. N= 5 cages per group, except 10 controls

2 ANOVA was done on log of data

Means without a common letter differ, $P<0.05$

Figure I: Formalin-fixed colon after HID-AB staining (original magnification, x32). A: Identification of a MDF of five mucin-depleted crypts B: Identification of a MDF of eleven mucin-depleted crypts.

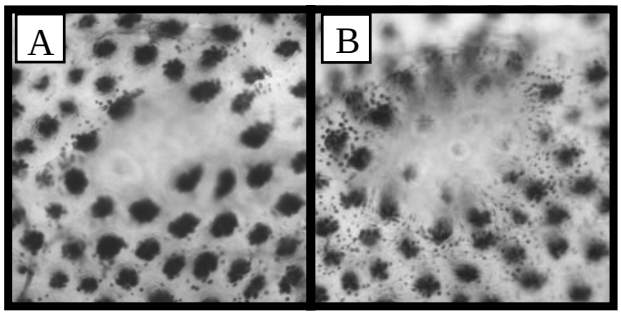
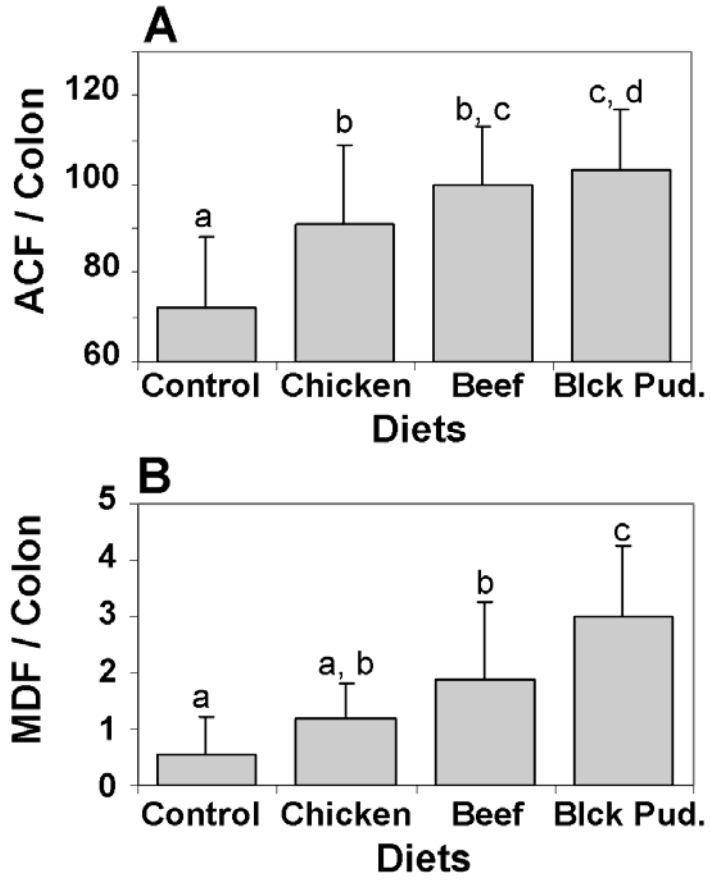


Figure 2: Number of putative pre-cancerous lesions per rat colon after 100 d on the experimental diets and one injection of azoxymethane. A: Number of aberrant crypt foci. B: Number of mucin-depleted foci. Data are means ± SD. N=10 rats/group, except 20 controls. Means without a common letter differ, P<0.05



References

1. WCRF & AICR (1997) Food, Nutrition and the prevention of cancer: a global perspective. Washington, DC: World Cancer Research Fund and American Institute for Cancer Research.
2. Norat, T., Lukanova, A., Ferrari, P. & Riboli, E. (2002) Meat consumption and colorectal cancer risk: dose-response meta-analysis of epidemiological studies. Int. J. Cancer 98: 241-256.
3. McIntosh, G. H., Regester, G. O., Leleu, R. K., Royle, P. J. & Smithers, G. W. (1995) Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. J. Nutr. 125: 809-816.
4. Clinton SK, D. R., Anderson DB, Truex CR, Imrey PB, Visek WJ (1979) 1,2-dimethylhydrazine induced intestinal cancer in rats fed beef or soybean protein. Nutr. reports Int. 20: 335-342.
5. Nutter, R. L., Gridley, D. S., Kettering, J. D., Goude, A. G. & Slater, J. M. (1983) BALB/c mice fed milk or beef protein: differences in response to 1,2-dimethylhydrazine carcinogenesis. J. Natl. Cancer Inst. 71: 867-874.
6. Reddy, B. S., Narisawa, T. & Weisburger, J. H. (1976) Effect of a diet with high levels of protein and fat on colon carcinogenesis in F344 rats treated with 1,2-dimethylhydrazine. J. Natl. Cancer Inst. 57: 567-569.
7. Pence, B. C., Butler, M. J., Dunn, D. M., Miller, M. F., Zhao, C. & Landers, M. (1995) Non-promoting effects of lean beef in the rat colon carcinogenesis model. Carcinogenesis 16: 1157-1160.
8. Pence, B. C., Landers, M., Dunn, D. M., Shen, C. L. & Miller, M. F. (1998) Feeding of a well-cooked beef diet containing a high heterocyclic amine content enhances colon and stomach carcinogenesis in 1,2-dimethylhydrazine-treated rats. Nutr. Cancer 30: 220-226.
9. Lai, C., Dunn, D. M., Miller, M. F. & Pence, B. C. (1997) Non-promoting effects of iron from beef in the rat colon carcinogenesis model. Cancer Lett. 112: 87-91.
10. Alink, G. M., Kuiper, H. A., Hollanders, V. M. H. & Koeman, J. H. (1993) Effect of heat processing and of vegetables and fruit in human diets on 1,2-dimethylhydrazine-induced colon carcinogenesis in rats. Carcinogenesis 14: 519-524.
11. Mutanen, M., Pajari, A. M. & Oikarinen, S. I. (2000) Beef induces and rye bran prevents the formation of intestinal polyps in apc(min) mice: relation to beta-catenin and PKC isozymes. Carcinogenesis 21: 1167-1173.
12. Kettunen, H. L., Kettunen, A. S. L. & Rautonen, N. E. (2003) Intestinal immune responses in wild-type and apc(min/+) mouse, a model for colon cancer. Cancer Res. 63: 5136-5142.
13. Parnaud, G., Peiffer, G., Tache, S. & Corpet, D. E. (1998) Effect of meat (beef, chicken, and bacon) on rat colon carcinogenesis. Nutr. Cancer 32: 165-173.
14. Belobrajdic, D. P., Mcintosh, G. H. & Owens, J. A. (2003) Whey proteins protect more than red meat against azoxymethane induced ACF in wistar rats. Cancer Lett. 198: 43-51.
15. Hakkak, R., Korourian, S., Ronis, M. J. J., Johnston, J. M. & Badger, T. M. (2001) Dietary whey protein protects against azoxymethane-induced colon tumors in male rats. Cancer Epidemiol. Biomarkers Prev. 10: 555-558.
16. Sesink, A. L. A., Termont, D. S. M. L., Kleibeuker, J. H. & Vandermeer, R.

(1999) Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme. Cancer Res. 59: 5704-5709.

17. Sesink, A. L. A., Termont, D. S. M. L., Kleibeuker, J. H. & VanDerMeer, R. (2001) Red meat and colon cancer: dietary haem-induced colonic cytotoxicity and epithelial hyperproliferation are inhibited by calcium. Carcinogenesis 22: 1653-1659.

18. Pierre, F., Tache, S., Petit, C. R., Van der Meer, R. & Corpet, D. E. (2003) Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. Carcinogenesis 24: 1683-1690.

19. Corpet, D. E. & Tache, S. (2002) Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. Nutr. Cancer 43: 1-21.

20. Caderni, G., Femia, A. P., Giannini, A., Favuzza, A., Luceri, C., Salvadori, M. & Dolara, P. (2003) Identification of mucin-depleted foci in the unsectioned colon of azoxymethane-treated rats: correlation with carcinogenesis. Cancer Res. 63: 2388-2392.

21. Femia, A. P., Dolara, P. & Caderni, G. (2004) Mucin-depleted foci (MDF) in the colon of rats treated with azoxymethane (AOM) are useful biomarkers for colon carcinogenesis. Carcinogenesis 25: 277-281.

22. American Inst. Nutr. (1977) Report of the American Institute of Nutrition. Ad Hoc Committee on standards for nutritional studies. J. Nutr. 107: 1340-1348.

23. Bird, R. P. (1987) Observation and quantification of aberrant crypts in murine colon treated with a colon carcinogen: preliminary findings. Cancer Lett. 37: 147-151.

24. Ohkawa, H., Ohishi, N. & Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem., 95:. 351-358.

25. Van den Berg, J. W., Koole-Lesuis, R., Edixhoven-Bosdijk, A. & Brouwers, N. (1988) Automating the quantification of heme in feces. Clin. Chem., 34: 2125-2126.

26. Govers, M. J. A. P., Termont, D. S. M. L., Lapre, J. A., Kleibeuker, J. H., Vonk, R. J. & Vandermeer, R. (1996) Calcium in milk products precipitates intestinal fatty acids and secondary bile acids and thus inhibits colonic cytotoxicity in humans. Cancer Res. 56: 3270-3275.

27. Bonneson, C., Eggleston, I. M. & Hayes, J. D. (2001) Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. Cancer Res. 61: 6120-6130.

28. Lapre, J. A. & Vandermeer, R. (1992) Diet-Induced Increase of Colonic Bile Acids Stimulates Lytic Activity of Fecal Water and Proliferation of Colonic Cells. Carcinogenesis 13: 41-44.

29. Sawa, T., Akaike, T., Kida, K., Fukushima, Y., Takagi, K. & Maeda , H. (1998) Lipid peroxyl radicals from oxidized oils and heme-iron: implication of a high-fat diet in colon carcinogenesis. Cancer Epidemiol. Biomarkers Prev. 7: 1007-1012.

30. Corpet, D. E., Tache, S. & Pierre, F. (2002-2004) Colon cancer prevention: chemoprevention database. http://www.inra.fr/reseau-nacre/sci-memb/corpet/indexan.html July 1, 2004.

31. Li, D., Ng, A., Mann, N. J. & Sinclair, A. J. (1998) Contribution of meat fat to dietary arachidonic acid. Lipids 33: 437-440.

32. McEntee, M. F. & Whelan, J. (2002) Dietary polyunsaturated fatty acids and colorectal neoplasia. Biomed. Pharmacotherapy 56: 380-387.

33. Kirkland, J. B. (2003) Niacin and carcinogenesis. Nutr Cancer 46: 110-118.

34. Morrow, J. D., Awad, J. A., Oates, J. A. & Roberts, L. J. (1992) Identification of skin as a major site of prostaglandin D2 release following oral administration of niacin in

humans. J. Invest. Dermatol., 98: 812-815.
35. Lee, D. H., Anderson, K. E., Harnack, L. J., Folsom, A. R. & Jacobs, D. R., Jr. (2004) Heme iron, zinc, alcohol consumption, and colon cancer: Iowa Women's Health Study. J. Natl. Cancer Inst. 96: 403-407.
36. Singh, P. N. & Fraser, G. E. (1998) Dietary risk factors for colon cancer in a low-risk population. Amer. J. Epidemiol. 148: 761-774.