Beef meat promotion of dimethylhydrazine-induced colorectal carcinogenesis biomarkers is suppressed by dietary calcium.

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**Key words**: Red meat, colorectal cancer, aberrant crypt foci, carcinogenesis promotion

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**Abbreviations**: ACF: aberrant crypt foci, MDF: mucin-depleted foci, TBARS: thiobarbituric
acid reactive substances, DHN-MA: 1,4-dihydroxynonane mercapturic acid
Abstract

Red meat consumption is associated with increased risk of colorectal cancer. We have previously shown that haemin, haemoglobin and red meat promotes carcinogen-induced preneoplastic lesions: aberrant crypt foci (ACF) and mucin-depleted foci (MDF) in rats. We have also shown that dietary calcium, antioxidant mix and olive oil, inhibit haemin-induced ACF promotion, and normalize faecal lipoperoxides and cytotoxicity. Here we tested if these strategies are effective also against red meat promotion in dimethylhydrazine-induced rats.

Three diets with 60% beef meat were supplemented with calcium phosphate (33 g/kg), antioxidant agents (rutin and BHA, 0.05% each), and olive oil (5%). ACF, MDF, faecal water cytotoxicity, thiobarbituric acid reactive substances (TBARS), and urinary 1,4-dihydroxynonane mercapturic acid (DHN-MA) were measured. Beef-meat diet increased the number of ACF (+30%) and MDF (+100%) (P < 0.001), which confirms our previous findings. Promotion was associated with increased faecal water TBARs (x4) and cytotoxicity (x2), and urinary DHN-MA excretion (x15). Calcium fully inhibited beef meat-induced ACF and MDF promotion, and normalized faecal TBARS and cytotoxicity, but did not reduce urinary DHN-MA. Unexpectedly, high-calcium control diet-fed rats had more MDF and ACF in the colon than low-calcium control diet-fed rats. Antioxidant mix and olive oil did not normalize beef-meat promotion nor biochemical factors. The results confirm that haem causes promotion of colon carcinogenesis by red meat. They suggest that calcium can reduce colorectal cancer risk in meat-eaters. They support the concept that toxicity associated with the excess of a useful nutrient may be prevented by another nutrient.
**Introduction**

Red and processed meat consumption increases the risk of colorectal cancer, according to meta-analyses of epidemiological studies\(^1\). Several explanations have been given for this increase, involving fat, heterocyclic amines, N-nitrosated compounds, and haem iron. Intake of dietary haem iron is associated with an increased risk of colon cancer among women of the Iowa Women's Health Study\(^3\). Intake of black pudding, a blood sausage high in haem iron, is associated with increased risk of colorectal cancer among women of the Swedish Mammography Cohort\(^4\).

Experimental animal studies support the hypothesis that haem iron promotes colorectal carcinogenesis\(^5\). We have shown that dietary haem, in the form of haemin, haemoglobin or red meat, promotes putative precancerous lesions, aberrant crypt foci (ACF) and mucin-depleted foci (MDF), in the colon of rats given a low-calcium diet\(^6\). Haemin is a free haem ring stabilized by a chlorine atom, in contrast with chlorine-free protein-bound haem in meat myoglobin. This haem-induced promotion is associated with increased lipoperoxidation and cytotoxicity of faecal water, and linked to urinary DHN-MA excretion, a lipid peroxidation biomarker\(^8\). The addition of calcium, antioxidant mix or olive oil to the diet inhibits haem-induced lipoperoxidation, cytotoxicity and promotion of carcinogenesis in rats\(^6\), but no demonstration has already been given that these dietary factors can inhibit promotion by red meat. We thus suggested, after van der Meer\(^9\), that diets high in calcium and oxidation-resistant fat could prevent promotion by red meat.

This study was designed to test the hypothesis that addition of calcium, antioxidant mix or olive oil to the diet can inhibit cancer-promoting effect of haem provided by beef meat.
Materials and Methods

Animals and Diets

Eighty Fischer 344 female rats were purchased at four weeks of age from Ifa Credo (St. Germain l'Arbresle, France). Animal care was in accordance with the guidelines of the European Council on animals used in experimental studies. The animal colony and staff got an official agreement #31-121 for in vivo rodents studies by French government. Rats were housed by pairs into stainless steel wire bottomed cages. The room was kept at a steady acclimatization to the animal colony and given control diet before being injected i.p. with the carcinogen 1,2 dimethylhydrazine (Sigma chemical, St. Quentin, France; 190 mg/kg body wt) in NaCl (9 g/L). Usually, several injections are given to rats. We reasoned that the first shot initiates carcinogenesis, and the following shots promote it, blurring diet-induced promotion.

We thus chose a single-shot protocol, following Glauert\textsuperscript{10}. Seven days later, rats were randomly allocated to eight groups of ten, and allowed free access to their respective diet for 100 days. We chose to initiate all rats with the carcinogen, since the study was designed to show dietary promotion, and because a 2.5% haemoglobin diet does not initiate ACF in rats (Pierre and Corpet, unpublished results).

Eight experimental diets shown in Table 1 were based on the diet fed to control rats, a modified AIN-76 powdered diet\textsuperscript{11} prepared and formulated in a powdered form by UPAE (INRA, Jouy-en-Josas, France). Dibasic calcium phosphate was included at a low concentration of 2.7 g/kg. Four beef meat diets were formulated to contain 60% meat (freeze-dried) w/w. Beef meat contained 0.6 µmol/g of haem. We chose this meat level, higher than what most people eat, to be able to detect protection against promotion. The effect of calcium and of an antioxidant mix was tested in two groups of rats given a beef meat diet supplemented either with calcium phosphate (31 g/kg) or antioxidant mix (butylated hydroxyanisole and rutin, 0.05% each). Last, the effect of oil was investigated by replacing safflower oil by extra-virgin olive oil (5%, Puget, France) in a beef meat diet. Because calcium, antioxidant mix and olive oil might be protective even in the absence of meat, three other groups received beef-free control diet, supplemented with calcium phosphate, antioxidant mix or olive oil. As shown on Table 1, all diets were balanced for protein (50%), fat (20%) and iron (110 mg/kg) by addition of casein, lard, and ferric citrate. The diets were made up every 14 days and maintained at −20°C to reduce lipoperoxidation.
ACF Assay

All 80 rats were killed by CO2 asphyxiation in a random order at day 99 or 100. Colons were excised from rats immediately post mortem, flushed with cold Krebs solution (Sigma chemical, St. Quentin, France), opened longitudinally and fixed flat between two sheets of filter paper in 10% formalin (Sigma chemical, St. Quentin, France). Eighty colons picked up in random order were stained for 6 min in a 0.05% filtered solution of methylene blue. Number of ACF per colon, and number of crypts in each ACF, were counted under light microscope at x40 magnification in duplicate by two readers, blinded for the origin of the colon. Data from the two readers were pooled.

MDF Assay

MDF may predict colon carcinogenesis better than ACF, since Apc mutations are present in MDF with a frequency similar to that of tumours. Colons, after being scored for ACF, were stained with high iron diamine-Alcian blue procedure (HID-AB) to evaluate mucin production. Briefly, colons were rinsed in distilled water and left overnight in freshly prepared HID solution (50 mL of distilled water with 120 mg N-N'-dimethyl-m-phenylene diamine, 20 mg N-N'-dimethyl-p-phenylene diamine, and 1.4 mL of 60% ferrie chloride). After rinsing, colons were counterstained in 1% alcian blue solution for 30 min. MDF number, and number of crypts per MDF, were scored blindly under light microscope at x40 magnification by a single reader.

Preparation of Faecal Water

Faecal pellets were collected under each cage of two rats for 24 h, thus leading to five samples per group. Freeze-dried faeces were used to calculate dry faecal mass and to prepare faecal water by adding 1 mL of sterilized water to 0.3 g of faeces. Samples were then incubated at 37°C for one hour, stirring thoroughly every 20 min, followed by centrifugation at 20 000g for 10 min. The aqueous phase was re-centrifuged at the same speed and duration and the subsequent supernatant (faecal water) collected and conserved at –20°C until use.

TBARS and Haem Assay

Thiobarbituric acid reactive substances (TBARS) were measured in faecal water according to Ohkawa et al., exactly as previously described. Haem contents of freeze-dried faeces and of faecal water were measured by fluorescence according to Van den Berg et al. and Sesink et al., respectively, as already described.
Cytotoxicity Assay of Faecal Water

Cytotoxicity of faecal water was quantified on a cell line according to Bonneson et al.\textsuperscript{17}, and as previously described\textsuperscript{7}. Briefly, cancerous mouse colonic epithelial cell line, CMT93 (ECAC), was seeded in 96-well microtiter plates (1.6 $\times$ 10$^4$ cells per well in 200 $\mu$L of medium) and treated for 24 h with faecal water sample diluted at 10% (v/v) in the culture medium. Cytotoxicity of each faecal water was quantified by the 3-(4,5-dimethyl-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) test.

Urinary DHN-MA assay

Each rat was placed alone in a metabolic cage, for two days during the fifth week of experimental diet. The 24-hour urine was collected under each cage of one rat, thus leading to ten samples per group. DHN-MA assay was done by competitive enzyme immunoassay (EIA) as previously described\textsuperscript{18}, using DHN-MA-linked acetylcholinesterase enzyme. Each urine sample was assayed in duplicate.

Statistical Analysis

Results were analyzed using Systat 10 software for Windows, and reported as mean $\pm$ SD. Values were considered firstly using one-way analysis of variance (ANOVA). If a significant difference was found between groups ($p<0.05$) then each experimental group was compared to the control group using the Fishers’s least-significant-difference test (ACF and MDF data), and the Dunnett multiple comparison test (all other data).
Results

Weight and food intake

Final body weight of rats was 198±3g, without significant differences between groups at the end of the experimentation. Food intake was the same in all groups of rats (data not shown).

ACF and MDF data

Beef meat diet doubled the number of MDF per colon compared to control rats, and increased the number of ACF per colon (both p<0.01, Fig. 1). Number of crypts per lesions also was enhanced by beef meat diet (Table 2).

Calcium fully suppressed beef meat-induced promotion of ACF and MDF (Fig. 1), but did not reduce the mean number of crypts per lesions (Table 2). Antioxidant mix and olive oil significantly reduced meat-induced promotion of MDF number, but did not normalize it to control values (Table 2). In contrast, antioxidant mix and olive oil did not interfere significantly with beef meat-induced promotion of ACF number (Table 2). Thus, only calcium supplementation fully suppressed the beef-induced promotion of ACF and MDF.

Supplementation of no-meat control diet with antioxidant mix or olive oil did not modify ACF or MDF incidence (Table 2), as previously observed \(^6\). In contrast, surprisingly, high-calcium control diet-fed rats had more MDF and ACF in the colon than low-calcium control diet-fed rats, with more crypts per lesion (p<0.01, Table 2).

Faecal haem, TBARS and Cytotoxicity

Haem intake and faecal haem values matched study design: as expected, little haem was detected in faeces of control rats, in contrast with beef meat-fed rats (Table 3). We also measured haem concentrations in faecal water because, according to bile acids studies, the soluble fraction of colonic contents would interact more strongly with the mucosa than the insoluble fraction. As expected, haem concentration in faecal water depended directly on haem level in beef-based diets (Table 3). However, rats fed the beef meat high-calcium diet had little haem in faecal water, as previously observed \(^6\).

Haem can induce the formation of peroxyl radicals in fats, which may be cytotoxic and cleave DNA \(in vivo\). Lipid peroxidation was thus measured in faecal water by TBARS assay. Lipid peroxidation correlated with haem concentration in faecal water (Table 3, r=0.95, p=0.0002). Calcium almost normalized beef-induced lipid peroxidation to control level (66 and 33 \(\mu\)M MDA equivalent, respectively), but antioxidant mix or olive oil did not reduce peroxide...
values (130 and 148 μM MDA equivalent, similar to 141 μM in beef meat-fed rats).

Furthermore, as already seen, faecal water of beef meat-fed rats was cytotoxic to CMT93 cells. Calcium fully inhibited beef meat-induced cytotoxicity of faecal water, but antioxidant mix and olive oil did not.

**Urinary DHN-MA Excretion**

Table 3 shows that beef meat-based diet increased urinary DHN-MA excretion by 15-fold compared with control diet \( (P<0.001) \). Calcium and olive oil did not reduce DHN-MA excretion in beef meat-fed rats, while antioxidant mix gave a slight decrease, though statistically significant.
Discussion

These data confirm that a red meat diet promotes colon carcinogenesis in rats. They also establish that red meat promotion can be suppressed by dietary calcium. In addition faecal water cytotoxicity and lipoperoxides level were linked with haem-induced promotion.

Red meat promoted ACF and MDF in the colon of carcinogen-induced rats, and meat promotion was associated with faecal water haem, cytotoxicity and TBARS, in line with our previous study. The mechanism of haem promotion is not known, and may be linked to the stimulation of the endogenous production of N-nitroso compounds. Alternatively, we suggest promotion is linked to peroxidation and cytotoxicity. We have explored the effect of faecal water from beef-fed rats on normal (Apc +/+ ) and premalignant colonic cells (Apc Min/+ ). Results show that Apc mutated cells survive haem-induced faecal lipoperoxides, notably 4-hydroxynonenal (HNE) that is toxic to normal cells. Selection of mutated cells by cytotoxic lipoperoxides may explain haem-promotion of colon carcinogenesis. Beef diet reduces caspase-3 activity in the rat colonic mucosa, thus decreasing apoptosis induction. In addition, loss of APC induces histone deacetylase: it is overexpressed in polyps of APC-deficient mice, and it prevents apoptosis of colonic cells. Furthermore, HNE alters histone acetylation. Therefore, the modification of histone acetylation by meat-induced HNE may reduce apoptosis induction and explain the selection of Apc-mutated cells. This speculation on meat-promotion mechanism is supported by the present study: ACF and MDF promotion in beef meat-fed rats was associated with high faecal lipoperoxides and cytotoxicity, and, as discussed below, dietary changes that normalized faecal lipoperoxides and cytotoxicity also suppressed beef meat-promotion.

Calcium added on top of beef-meat diet normalized number of ACF and MDF at the same low level as control group, a protection already observed against haemin-induced promotion. In faeces from rats given high-calcium beef meat diet, haem concentration was high (similar to beef meat-fed rats), but faecal water haem level was low (similar to no-meat control rats) (Table 3). Calcium phosphate precipitates haem in the gut, and little haem remains available to induce lipoperoxidation, as previously showed with haemin. Faecal water thus contained little lipid peroxides and showed little cytotoxic activity (Table 3). The trapping of haem by calcium abolished carcinogenesis promotion. This confirms that haem is the cause of red meat promotion. The results also suggest that calcium can reduce colorectal cancer risk in meat-eaters. It might explain discrepancies between clinical trials with calcium supplements, since results were not analyzed based on meat consumption by volunteers. This
could be looked for in a prospective cohort study, to know if the protective effect of calcium against red meat promotion can also be seen in human beings. Similarly, an epidemiological study shows that chlorophyll from vegetables attenuates the elevated risk of colon cancer associated with red meat intake.

No protection against beef meat promotion was afforded by the antioxidant mix or by olive oil (Table 2). These supplements did not normalize faecal values of haem lipoperoxides and cytotoxicity either (Table 3). These results conflicts with a previous study in which the antioxidant mix or olive oil had been effective against haemin-induced promotion. This conflict suggests that dietary haemin is not a suitable model for red meat to test preventive strategies in rodents. We speculate that antioxidant molecules added to the diet can directly prevent the effect of haemin powder on dietary oil. Here, in contrast, protein-bound haem in red meat might have been protected from the antioxidant effect. Furthermore, since faecal water lipoperoxides and cytotoxicity are associated with haem-induced carcinogenesis, here and in previous studies, we suggest they can be used as short term biomarkers to screen preventive strategies against red meat-induced promotion of colon cancer.

An unexpected result here was that rats eating a high-calcium control diet had more ACF and more MDF than low-calcium control diet-fed control rats. This promotion by calcium phosphate in a high-fat context was not seen in a low-fat context, in our previous haemin study. It is generally agreed that calcium reduces the risk of colorectal cancer, and a meta-analysis study shows that calcium modestly decreases tumour incidence in rats. However, in 14 studies out of 32, a non-significant tumour incidence increase was seen in calcium-fed rats. The meta-analysis also shows that some calcium salts are more protective than others, and that calcium phosphate affords no protection. Calcium phosphate (which we used here) sometimes promotes ACF or tumours. For instance Bull et al. showed that calcium phosphate promotes colon tumourigenesis in two genetic contexts (Sprague-Dawley and Fisher 344 rats). In both types of rat the promotion was more important in 30%-fat diet-fed rats than in 3%-fat diet-fed rats. Phosphate, not calcium, might be the promoting nutrient, an issue discussed by Bruce. To conclude, promotion by calcium phosphate in a high-fat context was unexpected, but has already been reported.

Another surprise here is that all beef meat diets increased urinary DHN-MA excretion more than ten-fold. We have recently proposed urinary DHN-MA as a noninvasive biomarker of haem iron-induced promotion of carcinogenesis. Indeed, DHN-MA excretion increases dramatically in rats given high-haem diets, and the excretion parallels ACF and MDF
numbers in azoxymethane initiated rats. However, in the present study, the addition of
calcium to the meat diet did not reduce DHN-MA excretion while it normalized ACF and
MDF promotion. DHN-MA is the major urinary metabolite of HNE, a lipid peroxidation
product. DHN-MA excretion reflects endogenous HNE formation in the body, together with
formation in the diet, the latter being probably quantitatively the most important, while
preneoplastic lesions such as ACF and MDF may be due to lipid peroxidation occurring
locally in the colon lumen. One can hypothesize that calcium blocks haem in the intestine but
not in the diet, because of its powdered form, so the overall DHN-MA excretion is not
significantly reduced by the addition of calcium although HNE formation is reduced in the
colon lumen. Indeed, in a previous work, we have shown that HNE was found three times less
in the faeces of rats fed beef + calcium diet as compared to beef diet. It thus seems that
DHN-MA is a biomarker of exposure to a promoting dose of haem in the diet, but DHN-MA
is not a risk factor. However, measurement of HNE in faeces, which can reflect HNE in colon
lumen, could be an indicator of the colon cancer risk associated with diets

In conclusion, this study shows for the first time calcium prevention of red meat
promotion of colon carcinogenesis. This study supports the concept that toxicity associated
with the excess of a useful nutrient like haeminic iron may be prevented by another nutrient
like calcium. Yoghurt or cheese can be eaten after red meat, provided portions are small
enough to avoid fat overload. We found hardly any example of similar antidote effect, except
may be the lactulose prevention of protein-induced hepatic encephalopathy. This calcium
preventive effect should be looked for in a cohort study by crossing haem and calcium intake
with adenoma or cancer risk. Furthermore, different haem forms do not have the same
promoting potency and cannot be antagonized by identical agents, since haemin effect is not
identical to red meat effect. Last, faecal water lipoperoxidation and cytotoxicity may be
responsible, at least in part, of haem-induced promotion of colon carcinogenesis.

ACKNOWLEDGMENTS
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Blas Y Estrada for the care of the animals.
References


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<td>128.6</td>
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\(^a\) Low-calcium casein
\(^b\) AIN76 mix, but 500g/kg of dibasic calcium phosphate replaced by sucrose in mineral mix
\(^c\) All diets contained 110 mg/kg iron. Iron concentration was measured in freeze-dried beef before preparing the diets. Other nutrients were balanced: 50% protein, 21% fat, 20-24% carbohydrate. (based on added components, no analysis was done on whole diets)
\(^d\) BHA, butylated hydroxyanisole
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 dimethylhydrazine.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Haem µmol/g diet</th>
<th>ACF/colon</th>
<th>AC/colon</th>
<th>Crypts/ACF</th>
<th>MDF/colon</th>
<th>MDC/colon</th>
<th>Crypts/MDF</th>
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<tr>
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<td>125b</td>
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<td>299c</td>
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<td>300c</td>
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<td>22</td>
<td>243a</td>
<td>48</td>
<td>2.3a</td>
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</table>

1 Values are means and SD, N = 10 rats/dietary group. Mean values within a column with unlike superscript letters were significantly different (P<0.05).
2 Diets were based on a low calcium formula, and were balanced for iron (110mg/kg). Each group had ten rats. See Table 1 for precise composition.
Table 3: Effect of meat-based diets on faecal values in rats, notably haem, lipoperoxides and cytotoxicity of faecal water.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Haem intake (µmol/d)</th>
<th>Dry faecal mass (g/d)</th>
<th>Haem in faeces (µmol/g)</th>
<th>Haem in faecal water (µM)</th>
<th>TBARS in faecal water MDA equivalents</th>
<th>Cytotoxicity on CMT93 cells % cells lysed</th>
<th>Urinary DHN-MA (ng/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12</td>
<td>0.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6</td>
<td>33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Beef</td>
<td>3.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.05</td>
<td>2.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>141&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Beef + Calcium</td>
<td>3.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.99&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.14</td>
<td>2.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Beef + Antiox.</td>
<td>3.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07</td>
<td>3.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>130&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
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<td>3.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11</td>
<td>3.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>148&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
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<td>0.10</td>
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<td>0.51&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
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<td>0.07</td>
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<td>7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39&lt;sup&gt;a&lt;/sup&gt;</td>
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1 Values are means and SD. Faecal values, N= 5 cages/group. Urine values, N=10 rats/group. Mean values within a column with unlike superscript letters were significantly different (P<0.05)

2 Diets were based on a low calcium formula, and were balanced for iron (110mg/kg). Each group had ten rats. See Table 1 for precise composition.
**Figure 1:** Effect of beef meat, and high-calcium beef meat, diets on putative precancerous lesions per rat colon 100 d after the injection of dimethylhydrazine. Top panel, A: Number of aberrant crypt foci (ACF). Bottom panel, B: Number of mucin-depleted foci (MDF). Values are means ± SD, N = 10. Means without a common letter differ, $P < 0.05.$

![Bar graph A](image)

**Diets**

- Control
- Beef
- Beef + Calcium

![Bar graph B](image)

**Diets**

- Control
- Beef
- Beef + Calcium