

**The Effect of Dietary Calcium Level on Heme Excretion in Rats Fed Beef:  
Relationship to Colon Carcinogenesis**

**A Work In Progress**

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**Chang Liu  
Daniel Gallaher, PhD  
Department of Food Science and Nutrition  
College of Food, Agricultural, and Natural Sciences  
University of Minnesota**

**Background:**

Colorectal cancer is the second most common cause of cancer death in affluent countries such as the United States (1). However, colorectal cancer is thought to be two different types of cancer. Colon cancer is cancer of the large intestine, the lower part of a human's digestive system, which differs from rectal cancer in that rectal cancer defines the same disease for the last several inches of the colon. This family of cancer is third most common cancer in both incidence and mortality, accounting for eleven percent of all cancer-related deaths.

For a small number of colorectal cancer cases, the primary cause is attributed to genetics (2). The remaining overwhelming majority of colon cancers are found to have arisen sporadically. In such cases, the main causal agent is an interaction with the patient's environment. Diet is the environmental factor that is most often studied. Meat, particularly beef, is a dietary factor that has received considerable scientific attention (3). Evidence supporting beef consumption as a risk factor for colon cancer is inconsistent, however. Nevertheless, a number of components of beef have been suggested to promote development of colon cancer, one of the most prominent being the heme content. Heme has been suggested to increase colon cancer risk by increasing oxidation in the large intestine, producing reactive oxygen species, which may damage the colonic mucosa. This damage would lead to an inflammatory response. Increases in inflammation in the colon are known to increase colon cancer risk (3).

Heme is a complex red organic pigment containing iron that is a component of the proteins hemoglobin and myoglobin. Heme is found in particularly high concentrations in red meats such as beef and blood sausage. A number of studies have reported an association between high levels of red meat consumption and an increased risk of colorectal cancer (4, 5). One such

study, conducted by Giovannucci et al., was a prospective cohort study of middle-aged and elderly men (4). The fat, meat, fiber, and vegetable intake of these men were examined in relation to risk of colon cancer. Information was collected via questionnaires on diet, lifestyle and other health-related factors. Giovannucci et al. found that intakes of total fat, saturated fat, and animal fat were not related to risk of colon cancer. No clear association was found between fiber or vegetable intake and risk of colon cancer. However, an elevated risk of colon cancer was associated with red meat intake. Specifically, men who ate beef, pork, or lam as a main dish five or more times per week had a relative risk of 3.57 compared to men eating these foods less than once per month. Another finding in this study was that the association with red meat was not confounded appreciably by other dietary factors, physical activity, body mass, alcohol intake, cigarette smoking, or aspirin use. Overall, this study found results that supported the hypothesis that intake of red meat is related to an elevated risk of colon cancer. Another study that found results supporting a similar conclusion was completed by Willett et al (5). This study was a prospective cohort study of women, with information collected through questionnaires. Evidence was found in the data collected for the hypothesis that a higher dietary intake of red meat and fat from animal sources increases the incidence of colon cancer.

However, there have also been numerous studies that show no such correlation between dietary red meat intake and incidence of colon cancer (6-9). In a study done by Nutter et al., rats were fed diets of protein from milk and/or beef (6). Results from this study were not conclusive towards any association between heme consumed in the diet and incidence of colon cancer. In another study conducted by Parnaud et al., the effect of the meats beef, chicken, and bacon in the diets of rats were observed on colon cancer prevalence (7). One hundred rats were randomized to ten different diets with adjusted fat and protein contents. The number of aberrant crypt foci

(ACF- an identified indicator for high colon cancer risk) was used to determine colon tumor promotion. The ACF was nearly the same in all groups except for the rats fed with bacon, which had decreased ACF multiplicity. Overall, the hypothesis that colonic heme can promote colon cancer is not supported by this study and that beef as a whole food does not promote growth of ACF and chicken does not protect against colon carcinogenesis. Dunn et al. also performed a study in this subject, specifically on rats on the effects of iron in red meat in the diet and incidence of colon cancer tumors (8). No significant differences were found in total incidence and number of colon tumors between the beef and control protein (casein) diets. These results demonstrate that, when lean beef is used as an iron source, the risk for colon carcinogenesis is not increased. One final study in this category was carried out by Khil et al (9). This study is similar to the other two addressed above in this paragraph, and found no relationship between number of ACF and the dietary components of the rats.

As a possible explanation for the inconsistency of the epidemiological studies showing an association between beef and colon cancer risk, it has been suggested that high dietary calcium may eliminate the cancer promoting effect of heme by binding to and causing precipitation of the heme, thus making it unable to interact with the colonic mucosa. An example of one such study is that was conducted by Sesink et al. (3). In this study, rats were fed diets containing different levels of calcium and heme, and their fecal water was then analyzed to determine cytotoxicity. Colonic epithelial proliferation was also measured. Increases in fecal water cytotoxicity and epithelial proliferation have both been used as indicators of an increase in the risk of colorectal cancer. They reported a large increase in cytotoxicity of the fecal water of the rats fed a low calcium and high heme-containing diet compared to that of the control group, but no increase in

cytotoxicity for fecal water of rats fed diets high in calcium and heme content. Furthermore, increased colonic epithelial proliferation was found in the rats fed the low calcium diet.

It is important to understand the effects of beef on colon cancer risk. Beef is a widely consumed food, and provides a source of high quality protein and is a major source of dietary iron. Without beef, it is quite difficult for many people to obtain their requirement of iron. If beef does increase colon cancer risk, but that risk can be mitigated by increasing dietary calcium, this would be exceedingly useful to know. Likewise, if beef does not increase colon cancer risk, then this knowledge would allow researchers to focus their efforts on other foods as modulators of colon cancer risk. Measuring fecal heme content will complete the story for this study and allow us to publish our findings regarding the relationship between beef, calcium, and colon cancer risk in a well-accepted animal model of colon cancer.

Professor Daniel Gallaher of the Department of Food Science and Nutrition in the College of Food, Agriculture, and Natural Resource Sciences (CFANS) has been studying dietary influences on colon cancer, using animal models (10). One of the experiments recently conducted in his laboratory investigates the associations between colorectal cancer risk and different levels of dietary calcium and beef, as a source of heme. The heme content in the large intestine has been proposed as the component of beef that promotes colon cancer risk. In this study the marker for colon cancer risk used was colonic precancerous lesions (aberrant crypts), which were expressed in three different ways: the total number of aberrant crypts, aberrant crypt foci, and large aberrant crypt foci (foci with 4 or more aberrant crypts). The results of the study to date found no simple relationship between the amount of beef in the diet and the risk of colon cancer. None of the aberrant crypt markers showed a positive linear correlation with increasing dietary heme. A striking result is that the measurements of the rats fed the low calcium, 15%

beef diet showed the highest number of aberrant crypts, and the difference between this diet and all others was large for all three expressions of the markers. If one were only looking at 15% beef diets, then this would suggest that dietary heme increases colon cancer risk, but that this risk can be reduced by increasing dietary levels of calcium. However, the diets with the highest level of heme – the 60% beef diets – did not differ in projected risk from the casein diets. This finding challenges the hypothesis that heme in the diet increases risk of colon cancer. Results were complicated and there was no simple conclusion drawn.

Overall, the findings from these studies evaluated were mixed. One observation of interest is that the studies that found data that supported the hypothesis that dietary heme promotes colonic carcinogenesis were all human prospective cohort studies. All studies that found no such association were experimental studies performed on rats. Perhaps these conclusions of the latter type of studies should not be drawn to apply to humans. Ideally, studies would be done without detriment to the health of any individuals but with high applicability to human health. However, as of yet there are no such studies that have been speculated or performed. Possible future studies could include performing animal experiments on other species with similar digestive physiology to humans. Because the research on calcium and its canceling effect on heme in the colon is few in number. Future studies could also focus on performing more of these types of studies to get a greater number of conclusions and thus a stronger understanding of this specific hypothesis.

**Objectives:**

This research project investigated the relationship between dietary calcium and heme with the prevalence and risk of colorectal cancer. It attempts to expand upon Dr. Gallaher's group's findings. The goal of the project was to determine two things: (1) the heme content in the fecal samples of the animals fed the different diets and (2) whether the heme content correlates with the number of precancerous lesions, the marker of colon cancer risk used in the study.

**Experimental Design & Methodology:**

This study used rats to model the effects of heme and calcium in the diet on colon cancer proliferation. The rats were adapted to a control diet for 5 days, then injected with the colon-specific carcinogen dimethylhydrazine with a dose proportional to their body weight, once a week for two weeks, and then fed the experimental diets for the next 10 weeks (3). Nine diets were used, and each based on the AIN-93G standard rodent diet. There was a control diet (0 heme), a 15% beef diet (low heme - 0.11  $\mu\text{mol/g}$  diet), and a 60% beef diet (high heme - 0.44  $\mu\text{mol/g}$  diet heme), each of which were fed with a low (20  $\mu\text{mol calcium/g}$  diet), moderate (50  $\mu\text{mol calcium/g}$  diet), and high (130  $\mu\text{mol calcium/g}$  diet) dietary calcium concentration. The dietary groups are shown in the table below.

<b>Heme</b> <b>Calcium</b>	<b>None</b> (0 $\mu\text{mol/g}$ diet)	<b>Low</b> (0.11 $\mu\text{mol/g}$ diet)	<b>High</b> (0.44 $\mu\text{mol/g}$ diet)
<b>Low</b> (20 $\mu\text{mol/g}$ diet)	<b>Low calcium casein diet</b>	<b>Low calcium 15% beef diet</b>	<b>Low calcium 60% beef diet</b>
<b>Moderate</b> (50 $\mu\text{mol/g}$ diet)	<b>Moderate calcium casein diet</b>	<b>Moderate calcium 15% beef diet</b>	<b>Moderate calcium 60% beef diet</b>
<b>High</b> (130 $\mu\text{mol/g}$ diet)	<b>High calcium casein diet</b>	<b>High calcium 15% beef diet</b>	<b>High calcium 60% beef diet</b>

The study used beef cooked over low heat to prevent formation of potentially carcinogenic compounds formed in beef at high cooking temperatures (e.g. heterocyclic amines) and freeze-dried before use. Proximate analysis of the beef was completed to balance the diets so that the protein, carbohydrate, dietary fiber, dietary fat, and phosphorous content for all diets were equivalent. Casein was added to the 15% beef-containing diets to give a 20% total protein concentration by weight. Calcium in the diets was given using dairy calcium, which has a minimum calcium content of >27%. Because dairy calcium contains a small amount of lactose (<10%), which is highly fermentable and thus could alter the colonic microbial environment, the amount of lactose was equalized across all diets to control for this potential effect.



To measure the prevalence of markers of colon cancer after the 10 weeks of diet regulation, the rats were anesthetized and their colons removed. For measurement of morphological markers, the colons were flushed with PBS, cut open along the longitudinal median, and fixed flat in 10% buffered formalin (1,3). Aberrant crypts (AC) and aberrant crypt foci (ACF) had already been counted as part of the project earlier conducted by Gallaher's group, on  $2 \times 5 \text{ cm}^2$  sections of the distal colon, and stained with methylene blue. To analyze cecal contents, they were collected from the cecum and centrifuged at  $30,000 \times g$  for 30 min at  $4^\circ\text{C}$ . The calcium concentration was also determined from the supernatant using a calcium-specific microelectrode.

This project involves the analysis of the fecal matter of the rats for heme concentration. The fecal matter was preserved before analysis (3). An acidified methanol/chloroform extraction (final HCl concentration 1 M) was carried out on 20 mg of fecal samples. The chloroform phase will be recovered after centrifugation and dried under nitrogen. The samples were then dissolved in 0.45 mL of 250 mM KOH, sonicated for 5 min and mixed with 0.45 mL of distilled water, 3.75 mL of 2-propanol and 0.75 mL of 1.15 M HCl. This mixture was homogenized, then centrifuged for 10 min at 1500 g, after which the supernatants were assayed for their heme content as follows: 50  $\mu\text{L}$  samples of the supernatants was mixed with 1 mL of glacial acetic acid. Subsequently, 50  $\mu\text{L}$  of freshly prepared 0.12 M  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 4.5 M HCl were added. Samples were then immediately mixed and incubated at  $60^\circ\text{C}$  for 30 minutes. Two milliliters of 2-propanol/water (1/1) were added before fluorescence measurement using excitation and emission wavelengths of 400 and 594 nm. Blanks were obtained using the same protocol, but without the incubation at  $60^\circ\text{C}$ .

Fecal heme concentration was then analyzed statistically by two-way analysis of variance, with dietary heme level and dietary calcium level as the two main effects, using SAS statistical software. Differences among individual groups were intended to be inspected using a multiple range test. Any correlations were determined between fecal heme concentration and the number of precancerous lesions.

### **Results & Conclusion:**

The project is still in progress. Data found thus far has not been statistically significant. Further replication of trials need to be conducted in order to make any findings relevant.

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