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Research Article

Cancer Prevention Research

Inhibition by Resistant Starch of Red Meat–Induced Promutagenic Adducts in Mouse Colon

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Abstract

Population studies have shown that high red meat intake may increase colorectal cancer risk. Our aim was to examine the effect of different amounts and sources of dietary protein on induction of the promutagenic adduct O^6-methyl-2-deoxyguanosine (O^6MeG) in colonocytes, to relate these to markers of large bowel protein fermentation and ascertain whether increasing colonic carbohydrate fermentation modified these effects. Mice ($n = 72$) were fed 15% or 30% protein as casein or red meat or 30% protein with 10% high amylose maize starch as the source of resistant starch. Genetic damage in distal colonocytes was detected by immunohistochemical staining for O^6MeG and apoptosis. Feces were collected for measurement of pH, ammonia, phenols, *p*-cresol, and short-chain fatty acids (SCFA). O^6MeG and fecal *p*-cresol concentrations were significantly higher with red meat than with casein ($P < 0.018$), with adducts accumulating in cells at the crypt apex. DNA adducts ($P < 0.01$) and apoptosis ($P < 0.001$) were lower and protein fermentation products (fecal ammonia, $P < 0.05$; phenol, $P < 0.0001$) higher in mice fed resistant starch. Fecal SCFA levels were also higher in mice fed resistant starch ($P < 0.0001$). This is the first demonstration that high protein diets increase promutagenic adducts (O^6MeG) in the colon and dietary protein type seems to be the critical factor. The delivery of fermentable carbohydrate to the colon (as resistant starch) seems to switch from fermentation of protein to that of carbohydrate and a reduction in adduct formation, supporting previous observations that dietary resistant starch opposes the mutagenic effects of dietary red meat. *Cancer Prev Res; 4(11); 1920–8. ©2011 AACR.*

Introduction

Colorectal cancer (CRC) incidence is high in developed countries, and dietary habits have been implicated as the main factor in determining CRC risk. The World Cancer Research Fund recently identified red meat as a convincing cause of CRC, with the suggestion that intake more than approximately 500 g of cooked weight per week is associated with significantly increased risk of CRC (1). Prospective human studies have shown that increasing red or processed meat intake significantly increases the risk of CRC, particularly in the distal colon (2–5). However, greater consumption of protein intake as white meat, such as fish or chicken, is not associated with increased CRC risk (2, 6). Recent studies from animal models *in vivo* have shown that high dietary protein can increase DNA damage as single- and double-strand breaks, measured by the comet assay, particularly with red (but not white) meat intake (7, 8). These data support the findings of prospective population studies, but the mechanisms by which increasing red meat intake can increase CRC risk is not completely understood. A number of mechanisms have been proposed. These include increased *N*-nitroso compounds (NOC) forming DNA adducts due to excess amino acids and peptides in the colon (9), increased dietary heme from hemoglobin in red meat forming reactive oxygen species (10), and increased mutagenic compounds such as heterocyclic amines from cooking red meat at high temperatures (11). Furthermore, it is broadly implied that high protein diets increase protein bacterial fermentation of red meat in the colon, which might play a pivotal role in altering the biomarkers associated with CRC formation (12).

Dietary fiber is thought to exert a protective effect in the colon. In 1971, Burkitt proposed that dietary fiber decreases the incidence of CRC. This was based on comparisons of apparent differences in fiber intake between low- and high-risk populations. However, subsequent epidemiologic studies have given conflicting results (13–15). One possible reason is a misunderstanding of the relative contribution of the polysaccharides that contribute to total dietary fiber. Nonstarch polysaccharides (NSP) resist small intestinal digestion completely and enter the large bowel. Resistant starch is the component of dietary starch that escapes

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digestion in the small intestine, and in the large bowel, it (plus a variable fraction of NSP) is fermented by the microflora. This fermentation increases the production of short-chain fatty acids (SCFA; ref. 16) and reduces toxic products of microbial protein fermentation (17). Compared with other fiber sources that reach the colon, resistant starch favors the production of the SCFA butyrate (18). Butyrate is particularly important for large bowel function. It is a major energy source for colon epithelial cells. *In vitro* studies using transformed colon cancer cell lines have shown that butyrate increases apoptosis (19), decreases cell proliferation, and increases differentiation (20). Animal studies *in vivo* show that butyrate-generating fermentable substrates increase the acute apoptotic response to a chemical carcinogenic insult and halt the cell cycle to allow for repair or removal of damaged cells (21). However, in normal epithelial cells *in vivo* (without chemical carcinogenic insult) increased butyrate level has been shown to lower baseline apoptosis, increase crypt height, and maintain normal cellular proliferation in the colon (22–24).

Although a majority of the evidence from rodent models supports a protective effect of resistant starch against CRC and that red meat consumption promotes DNA damage, there still remains some doubt. In addition, no data are available from animal models identifying dietary protein-induced early DNA adducts that are known to cause genetic instability if left unrepaired in the cell. The DNA adduct *O*^{6}-methyl-2-deoxyguanosine (O^6MeG) is a known mutagenic lesion in both animals and humans resulting from exposure to alkylating agents. If O^6MeG is left unrepaired in the cell, then a GT mismatch occurs after 1 round of replication; if this mismatch is not repaired, a G → A transition can arise following a second round of replication in the *K-ras* gene, a known mechanism of human oncogene activation and tumor suppressor gene inactivation (25). Red meat has been suggested to increase DNA alkylation in the colon via increased endogenous N-nitrosation, although little is known about DNA adduct formation in the colon in response to red meat consumption, particularly O^6MeG (26). Our aim was to examine the effect of different amounts and sources of dietary protein on induction of the promutagenic adduct O^6MeG, to relate these to markers of protein fermentation and ascertain whether increasing colonic carbohydrate fermentation modified these effects.

Materials and Methods

Animals and diets

A total of 72 eight-week-old wild-type CBJ57 male mice were obtained from the Animal Resource Centre, Perth, Western Australia. The Flinders University of South Australia Animal Welfare Committee approved all experimental procedures. Animals were randomly placed 4 mice per cage and divided into dietary groups under controlled conditions of 22°C (SD = 2°C), 80% ± 10% humidity, and 12-hour light/dark cycle. Animals had free access to food and water and were weighed once weekly throughout the study period.

The experimental diets were based on the American Institute of Nutrition (AIN) diet AIN-76, with modified amounts of vitamins and minerals for rats and mice adapted from the work of Newmark and colleagues (ref. 27; Table 1). The AIN-76 standard diet contains 20% casein, with a total protein content of 18.4%. Our experimental diets were a modification of the AIN-76 diet to give 15% and 30% of total protein and 30% protein with 10% high amylose maize starch (HAMS) and are summarized in Table 1.

Table 1. Composition of experimental diets (g/100 g in diet)

	Low casein	High casein	Low casein + RS	Low meat	High meat	High meat + RS
Casein[a]	17.6	35.3	35.3	—	—	—
Red meat[a]	—	—	—	20.43	40.9	40.9
Corn starch	10	10	—	10	10	—
High amylose maize starch	—	—	10	—	—	10
Sucrose	45.48	27.78	27.78	46.39	29.69	29.68
Sunflower seed oil	12.5	12.5	12.5	12.5	12.5	12.5
Lard[b]	7.5	7.5	7.5	3.76	—	—
α-Cellulose	2	2	2	2	2	2
L-Cysteine	0.3	0.3	0.3	0.3	0.3	0.3
Choline	0.12	0.12	0.12	0.12	0.12	0.12
Minerals[c]	3.5	3.5	3.5	3.5	3.5	3.5
Vitamins[c]	1.0	1.0	1.0	1.0	1.0	1.0

[a]Enough casein and red meat were added to reach a total protein content of 15% for the low protein diets and 30% for the high protein diets.
[b]Lard was added to the casein and low red meat diets to balance each diet for saturated fat and to give a total fat content of 20%.
[c]AIN-76 vitamin and mineral mixtures with modified calcium at 0.5 mg/g, phosphorus at 3.6 mg/g, folic acid at 0.23 μg/g, and vitamin D3 at 0.11 IU/g.

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Casein was added at 17.6% and 35.3% of the low and high casein diets, respectively. Red meat was added at 20.43% and 40.9% of the low and high red meat diets, respectively. HAMS (Hi-maize 260), type RS2, was used as the source of resistant starch and was supplied by the National Starch and Chemical Company. Hi-maize 260 has been shown to contain approximately 50% resistant starch (28) and was added at a level of 10 g/100 g diet; therefore, a total of 5% resistant starch was added to the diet. Lean, minced rump steak was purchased, cooked at medium temperature on a gas hot plate with continuous mixing to prevent the meat from burning, and oven dried overnight before grinding to powder. Total nitrogen level of the cooked and dried red meat was determined by the Dumas method (29), and the total protein content was calculated to 75%. Saturated fat content of the red meat was 6% and analyzed by standard fat extraction method (30). Moisture content of the meat was found to be 10% by weighing known amounts of meat product and drying overnight to calculate moisture lost from the sample. Final diet preparations were placed into air-sealed containers and stored at 4°C, with fresh food in the mouse cages replaced daily.

Fecal analysis

After 3 weeks on experimental diets, fresh fecal samples were collected. For measurement of fecal pH and ammonia, feces were placed into 3 times the weight to volume of cold saline, vortexed briefly, and pH measured with a glass-embodied electrode (Eutech Instruments). The remaining solution was centrifuged for 10 minutes at 3,000 rpm. A 100-μL supernatant aliquot was diluted to make a 1 in 3 dilution for ammonia measurement with the Janus Robotic Liquid handling System. Results were calculated as micromoles per liter in the original diluted fecal sample in saline.

Another fresh fecal sample was placed into 3 times the weight to volume of a SCFA/phenol internal standard (800 mL Milli-Q water, 120 μL heptanoic acid, and 50 mg *o*-cresol, pH 7.0). Two separate 100 μL aliquots were used to measure for 3 SCFAs (butyrate, acetate, and propionate) and for phenols and *p*-cresols. For SCFAs, 100 μL was injected into an Agilent Technologies 6890N Network Gas Chromatograph system fitted with a Zebron ZB-FFAP column (0.53×30 mm^2) and measured as described previously (31). For phenols and *p*-cresol, another 100 μL of distillate was injected into a Shimadzu LC-10AD HPLC machine, with RF-10AXL fluorescence detector set at excitation 284 nm, emission 310 nm, and measured as described previously (32).

Sample collection

After 4 weeks on experimental diets, the mice were anesthetized with a 10% ketamine and 10% metotomodine solution at 75 mg/kg and decapitated. After dissection, the distal colon was removed and placed into a 10% buffered formalin solution containing 3.6% formaldehyde for 24 hours and transferred to 70% ethanol for histologic processing. Tissue was rehydrated through gradient alcohols and embedded in paraffin wax.

Cell proliferation

Proliferative activity of distal colonic epithelial cells was measured with an antibody specific for the nuclear proliferating antigen Ki-67 (Dako) in combination with an immunochemical detection method in paraffin-embedded sections, as used previously (33). Slides were visualized under a light microscope by brown nuclear staining, and proliferation was assessed as the Ki-67–positive cells per crypt column length. All slides were independently and randomly coded.

Apoptosis measurement by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay

The level of apoptosis was measured by a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay kit (Trevigen) in paraffin-embedded sections, following the manufacture's instructions. Slides were visualized under a light microscope by brown nuclear staining, and apoptosis in the distal colon was assessed as the number of positive apoptotic nuclei per crypt column length. All slides were independently and randomly coded.

O^6MeG quantification

The level of DNA alkylation was quantified with an antibody specific for the DNA adduct O^6MeG combined with a horseradish peroxidase polymer (Poly-HRP) detection system on paraffin-embedded distal colon sections. Sections (4 μm) were cut, rehydrated, and incubated with 3% H_2O_2 in 50% ethanol for 15 minutes at room temperature. Antigen retrieval with 0.01 mol/L citrate buffer, pH 6.5, was carried out for 1 hour in a 2100 antigen retriever (PickCell Laboratories). RNase treatment [20 μL of RNase A at 10 mg/mL, 5 μL of RNase T at 10 U/mL, 1,000 μL PBS (pH 7.5)] was carried out for 1 hour at 37°C and stopped with a 5-minute treatment of 140 mmol/L NaCl solution at 4°C. DNA unwinding was achieved by an alkali treatment (1,500 μL of 70 mmol/L NaOH/140 mmol/L NaCl, 1,000 μL absolute methanol) before applying Special Block A (Covance Laboratories) for 30 minutes. The O^6MeG primary monoclonal antibody (clone EM 2-3; Squarix Biotechnology) was diluted to 1:1,000 in PBS and applied to the slides overnight at room temperature. The next day, slides were incubated with Special Block B for 30 minutes before applying the poly-HRP anti-mouse IgG (Covance Laboratories). Positive cells were visualized with DAB chromagen with substrate buffer (Signet Laboratories), counterstained with hematoxylin, dehydrated, and cover slipped for observation under a light microscope. Distal colon taken from a rodent injected with azoxymethane at 10 mg/mL and sacrificed 6 hours later was used as a positive stain control. The primary antibody was omitted from the protocol as a negative control.

Quantification of the positively stained nuclei was carried out by computer image analysis. Distal colonic crypts were selected for image analysis because that they had an open lumen with an intact single row of epithelial cells on each side. Twenty appropriate crypts were visualized under an Olympus BX-41 microscope, and images were captured

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Table 2. Effect of dietary protein amount, protein type, and resistant starch supplementation on fecal fermentation measures[a]

	Treatment groups						P[b]		
	Low casein	High casein	High casein + RS	Low meat	High meat	High meat + RS	Protein amount	Protein type	RS
Fecal pH	7.8 ± 0.1	7.5 ± 0.1	6.9 ± 0.6	7.2 ± 0.1	7.6 ± 0.1	6.7 ± 0.1	0.91	0.09	<0.0001
Acetate, μmol/g	16.8 ± 2.8	20.0 ± 3.4	28.0 ± 3.6	28.1 ± 4.6	25.3 ± 4.7	41.4 ± 3.9	0.76	0.07	<0.0001
Propionate, μmol/g	4.7 ± 0.6	4.9 ± 0.6	17.9 ± 2.2	6.4 ± 0.7	6.9 ± 0.9	26.4 ± 2.1	0.21	0.01	<0.0001
Butyrate, μmol/g	2.9 ± 0.6	3.5 ± 0.4	12.2 ± 1.7	3.9 ± 0.6	6.2 ± 1.7	17.4 ± 2.1	0.15	0.08	<0.0001
Total SCFAs, μmol/g	24.0 ± 3.8	28.4 ± 4.3	58.1 ± 7.0	38.4 ± 5.6	38.4 ± 6.2	85.2 ± 7.2	0.91	0.039	<0.0001
Ammonia, μmol/mL	6.5 ± 1.5	8.3 ± 1.9	4.5 ± 0.9	4.1 ± 0.3	6.3 ± 1.6	3.7 ± 0.4	0.19	0.15	0.05
Phenol, μg/g	1.5 ± 0.3	1.4 ± 0.3	0.2 ± 0.1	1.3 ± 0.3	1.9 ± 0.3	0.9 ± 0.3	0.29	0.53	<0.0001
p-Cresol, μg/g	3.8 ± 0.4	3.9 ± 0.8	2.2 ± 0.1	5.9 ± 0.9	7.4 ± 0.8	6.2 ± 0.6	0.48	<0.0001	0.16

Abbreviation: RS, resistant starch.

[a]Values expressed as means ± SE ($n = 12$).

[b]Univariate ANOVA was used to determine the main effect of protein amount, protein type, and resistant starch supplementation.

with an Olympus micropublisher 3.3 RTV camera and Olysia Bio-report software (Olympus). Camera and microscope settings were calibrated before each image to ensure consistency between images. To identify a linear path through a single row of nuclei along the crypt axis for all images taken, image analysis software developed by CSIRO Mathematics Informatics and Statistics division, "Imview" and "R for windows" 2.1.0, was used. Raw color (red, green, and blue), luminescence (L), normalized color values (r = red/L, g = green/L, and b = blue/L), and color ratio (RoB = r/b) data points were calculated for each pixel along the length of the linear path. The number of cells within each half crypt was counted, and the calculated RoB ratio was then averaged for each nucleus within individual crypts. Total O^6MeG values per crypt were achieved by the summation of the ratio value for each nucleus along the crypt axis. Distribution of adducts along the length of the crypt was quantified cell by cell. Total sum of adduct formation was calculated for each mouse and averaged for each dietary treatment. All slides were independently and randomly coded so that dietary groups were not known to the counter.

Statistical methods

Values are represented as means and SE. Univariate ANOVA was used to determine the main effect of protein amount (15% vs. 30%), protein type (casein vs. red meat), and resistant starch supplementation. Correlations between variables were identified by bivariate, one-tailed, parametric correlation tests. The null hypothesis was rejected at the 0.05 level for all statistical tests carried out.

Results

Body weight

No significant differences of body weight (in grams) were observed between experimental diets.

Fecal analysis

The effect of protein type, protein amount, and resistant starch supplementation on fecal measures are summarized in Table 2. There was no significant difference between 15% and 30% protein diets on any of the fecal variables (Table 2). Red meat significantly increased concentration of the protein metabolite *p*-cresol ($P < 0.0001$) and the carbohydrate fermentation products propionate ($P < 0.010$) and total SCFAs ($P < 0.039$) compared with casein. Resistant starch supplemented in the diet lowered fecal pH, ammonia, and phenol concentrations ($P < 0.05$) and increased all SCFAs ($P < 0.0001$).

Epithelial kinetics

Neither the amount nor the type of protein had an effect on cell proliferation, cell mass (crypt height), or rate of apoptosis, whereas resistant starch significantly increased proliferation and cell mass and reduced apoptosis (Table 3).

O^6MeG

DNA alkylation in the distal colon measured as the sum of average O^6MeG staining intensity (RoB ratio) in each colonic crypt was significantly higher in those mice consuming red meat than casein ($P < 0.018$), but amount of protein in the diet did not influence levels ($P < 0.92$; Fig. 1). Resistant starch supplementation significantly reduced such DNA adduct formation ($P < 0.01$; Fig. 1). Staining intensity was shown to increase from the base of the crypt toward the top of the crypt for all dietary treatments (Fig. 2).

Relationship between fermentation products and other biological events

Table 4 shows that there is a significant positive relationship between DNA adduct formation and levels of the protein fermentation product *p*-cresol ($P < 0.049$) and fecal pH ($P < 0.002$) but an inverse trend with concentration of the starch fermentation product butyrate ($P < 0.074$). Crypt

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Table 3. Effect of dietary protein amount, protein type, and resistant starch supplementation on distal colon epithelial cell kinetics[a]

	Treatment groups						P^b		
	Low casein	High casein	High casein + RS	Low meat	High meat	High meat + RS	Protein amount	Protein type	RS
Crypt height (cells)	22.3 ± 0.5	21.9 ± 0.6	23.1 ± 0.4	22.5 ± 0.6	22.3 ± 0.4	23.2 ± 0.4	0.62	0.59	0.03
Proliferation (cells per crypt)	3.7 ± 0.2	3.3 ± 0.2	3.7 ± 0.3	3.5 ± 0.2	3.5 ± 0.1	4.2 ± 0.4	0.39	0.85	0.04
Apoptosis (cells per crypt)	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.03	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.02	0.37	0.73	0.001

Abbreviation: RS, resistant starch.

[a]Values expressed as means ± SE ($n = 12$).

[b]Univariate ANOVA was used to determine the main effect of protein amount, protein type, and resistant starch supplementation.

height positively correlated with total SCFAs ($P < 0.05$). Fecal propionate ($P < 0.001$), butyrate ($P < 0.013$), and total SCFAs ($P < 0.01$) showed a significant inverse correlation with apoptosis in the distal colon. Apoptosis also showed a positive correlation with fecal pH ($P = 0.068$).

Discussion

This study showed that DNA adducts and concentrations in feces of compounds resulting from bacterial fermentation are influenced by dietary protein. There was a direct correlation between DNA adduct formation (O^6MeG) and levels of the protein fermentation product *p*-cresol, a substance considered to be genotoxic (34). The effects of protein on the formation of the promutagenic adduct O^6MeG in colonic epithelial cells were dependent more on type than on amount when comparing red meat with casein. This effect was evident in the context of comparing 2 levels of protein (i.e., 15% and 30%). When 10% HAMS (equivalent to 5% resistant starch) was added to the high protein diets (an achievable amount for human consumption), the effects of protein type were modulated; adduct formation, apoptosis, and fecal products of protein fermentation were lowered, whereas production of butyrate was increased. This study is the first to identify induction of a known promutagenic alkylating lesion in colonic DNA after short periods of feeding of red meat without chemical carcinogenic intervention, their reduction by resistant starch and

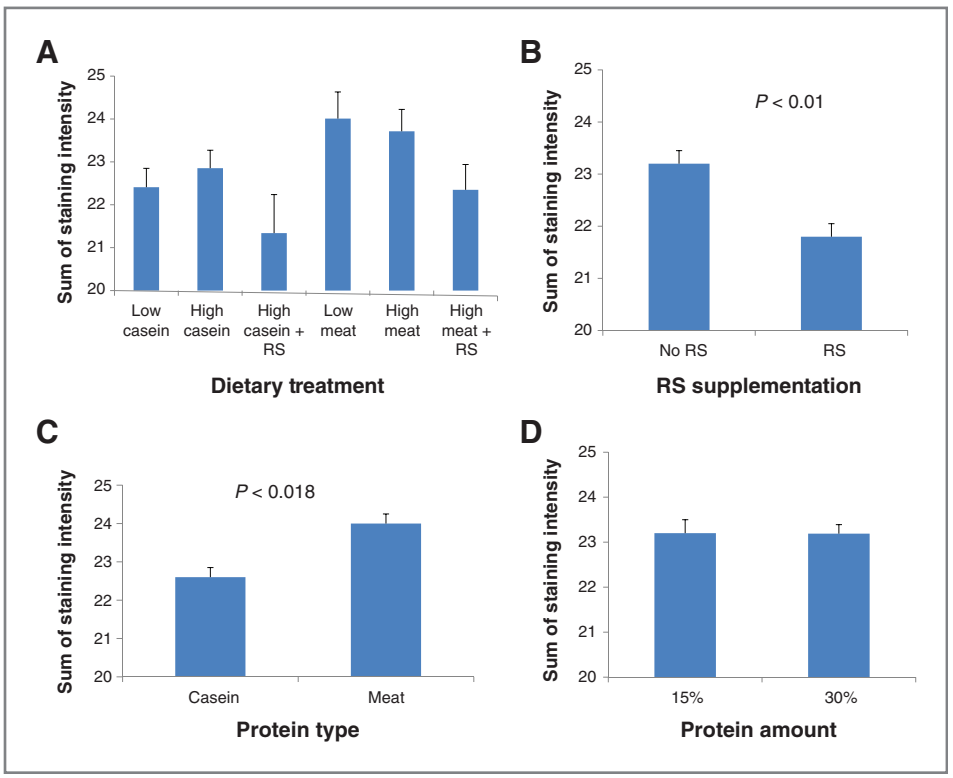

Figure 1. The average sum of O^6MeG staining intensity for each dietary treatment (A). Resistant starch (RS) supplemented in the diet (B) significantly reduced DNA adduct formation ($P < 0.01$) as compared with those diets without resistant starch. Compared with casein, red meat (C) significantly increased adduct formation ($P < 0.018$) irrespective of protein amount. There was no effect of protein amount (D) on DNA O^6MeG staining intensity irrespective of the protein source. Data are presented as means ± SE.

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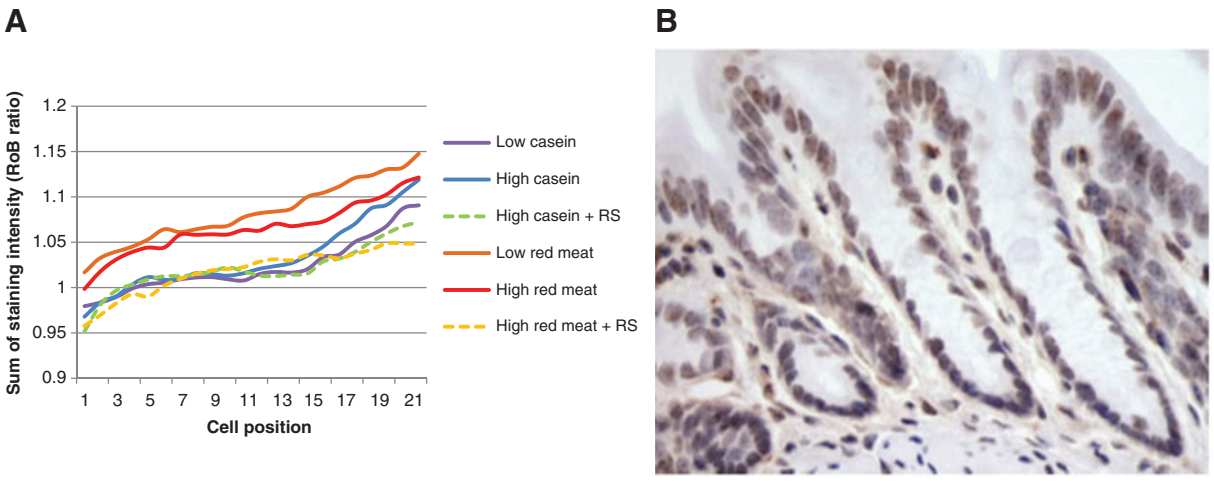

Figure 2. Average distribution of O^6MeG formation (A) for each dietary treatment along the length of the crypt. The highest cell number represents the top of the crypt. Data represented as RoB ratio, a higher value represents a higher intensity of positive staining in the cell nucleus. Light microscopic image of distal colonic crypt (B) at 20× optical zoom showing distribution of O^6MeG staining intensity. RS, resistant starch.

their distribution along the length of the crypt. However, other studies have shown similar effects for a number of protein sources on DNA single- and double-strand breaks. All of these data support the view that genomic stability is influenced negatively by increased dietary protein.

There were no changes in fecal pH or carbohydrate and protein fermentation products when increasing protein intake with either protein source. Protein fermentation products might be expected to increase on high protein diets compared with low protein diets, as some extra protein might reach the colon (12). However, others have reported that increasing protein intake as red or white meat in rats over a similar intervention period had no effect on the formation of phenols and *p*-cresols or on fecal pH (7). Toden and colleagues did show a significant increase in *p*-cresol in rats fed a 25% casein diet versus 15% casein diet after 4 weeks (35). Also, rats fed low and high protein diets over 5 months showed a significant increase in ammonia and fecal pH, which correlated with a higher proliferation rate in colonic epithelial cells (36). It is difficult to explain the variation between studies. Perhaps, changes in bacterial populations and metabolism had not yet stabilized within the colonic microbial population after 3 weeks in the mouse colon whereas it

Table 4. Relationships between fermentation products, epithelial kinetics, and adduct formation[a]

	Fecal pH	Ammonia	*p*-Cresols	Phenols	Acetate	Propionate	Butyrate	Total SCFAs
Crypt height								
Pearson correlation	−0.206	−0.130	−0.079	−0.100	0.240	0.215	0.114	0.244[b]
P	0.09	0.30	0.52	0.41	0.05	0.09	0.40	0.05
Proliferation								
Pearson correlation	−0.024	−0.159	−0.026	−0.194	0.059	0.197	0.177	0.163
P	0.85	0.21	0.83	0.11	0.64	0.12	0.18	0.20
Apoptosis								
Pearson correlation	0.224	−0.175	−0.015	0.161	−0.199	−0.362[c]	−0.325[b]	−0.320[b]
P	0.07	0.16	0.90	0.19	0.11	0.003	0.013	0.01
Sum of O^6MeG								
Pearson correlation	0.377[c]	−0.064	0.234[b]	0.046	0.029	−0.214	−0.234	−0.096
P	0.002	0.61	0.049	0.70	0.81	0.09	0.07	0.45

[a]Correlation for fecal measures versus DNA adducts and epithelial cell kinetics was undertaken by Pearson bivariate, one-tailed, parametric correlation tests.
[b]Significant at $P < 0.05$.
[c]Significant at $P < 0.01$.

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does seem to do so in the rat. The World Cancer Research Fund (1) suggests limiting red meat intake to 500 g of cooked weight per week, which is approximately 7% of the diet of a human consuming 1 kg of food per day. Experiments using protein at less than 15% are warranted to fully explore dose-dependent changes in the mouse.

Red meat was associated with significantly more *p*-cresols in the feces of mice than in a casein diet. Increases in fecal *p*-cresol concentration have been observed previously with red meat compared with white meat diets (7). These results might be explained by the digestibility of the different proteins. Casein is a highly digestible protein source with a true digestibility of 98% (37), and as a result, it is likely that little protein reaches the colon for bacterial fermentation. Cooked red meat as beef has a true digestibility of 90% (38), resulting in greater delivery of protein to the colon; this seems likely to account for the increased *p*-cresol levels.

Adding resistant starch to the diet profoundly altered protein and carbohydrate fermentation products in feces in a manner that can be interpreted as constituting a more favorable luminal environment. The addition of resistant starch to high protein diets reduced fecal pH, lowered production of phenols and ammonia, and increased fecal acetate, propionate, and butyrate concentrations. These changes may have certain benefits in relation to CRC risk. A low pH is considered to be associated with a reduced risk from CRC (39), whereas butyrate changes the metabolic environment in the lumen to promote a normal cellular phenotype (28). The addition of resistant starch to a high protein diet has been previously shown to reduce the formation of certain metabolites of protein fermentation (7). This is most likely a result of the bacteria favoring carbohydrate metabolism over protein when carbohydrate is readily available in the colon (40).

Our results showed that the type of protein consumed rather than the amount was the contributing factor in increasing DNA adducts in the distal colon, a key intermediate step toward DNA damage, cancer, and death. Red meat significantly increased the formation of O^6MeG when compared with casein. This might contribute to the mechanisms by which red meat increases risk for developing CRC in humans (1). Although O^6MeG is not the most predominant alkyl DNA adduct in number, in terms of biological significance, it is causing point mutations and mismatch repair recombination or cell death, with capacity to generate malignant transformation (25). There are no previous reports of the effect of dietary protein on the alkyl-induced DNA adduct O^6MeG. In a case study examining biopsies of normal colorectal mucosa, red meat was found to be a risk factor in the formation of N^7-methylguanine (N^7-MeG) but that adduct is not oncogenic (41). A randomized crossover trial comparing high red meat, vegetarian, and high red meat/high fiber diets showed marked increases of the O^6-carboxymethylguanine (O^6CMG) in exfoliated colon cells when healthy volunteers consumed a high red meat diet (9); however, exfoliated cells might acquire new adducts in the lumen with more direct exposure to protein fermentation products. Furthermore, evidence that O^6CMG can initiate oncogenesis is lacking (42). Mutation spectrums in functional yeast assays containing human p53 cDNA have indicated that O^6CMG is potentially promutagenic because potassium diazoacetate and *N*-methyl-*N*-nitrosourea produce differing mutation spectra; such adducts are also present in exfoliated colonic epithelial cells (43). Our study adds to existing information that when taken together confirms that red meat consumption results in increased formation in colonic epithelium of 3 adducts—N^7-MeG, O^6CMG, and now O^6MeG, the last being promutagenic. It is important to further investigate whether lower levels of red meat to that used in this study also affect adduct formation.

In this study, an increase in O^6MeG adducts correlated positively with fecal concentrations of *p*-cresol. Protein metabolites are known to be toxic, but relevance to CRC is debatable (36, 44, 45). *p*-Cresol could be playing a direct role in this model in inducing DNA damage, but limited data support this. Treatment of HL-60 cultured cells *in vitro* with *p*-cresol results in dose-dependent formation of various DNA adducts (46). A preliminary human study in patients with CRC measuring urinary *p*-cresol concentrations showed no difference compared with those of normal patients (34), although it is not clear whether urinary levels reflect colonic generation of this fermentation metabolite. Volunteers in a randomized intervention trial comparing a vegan diet with no meat or a standard diet including meat did show urinary and serum phenol and *p*-cresol levels to be higher with the meat diet (47). Increases in fecal *p*-cresol observed in red meat groups in this study show that their formation is linked to DNA damage measured as O^6MeG adducts, but it is not yet clear whether they are the direct initiating chemical or a marker that the damage is due to protein fermentation. It is possible that additional factors associated with red meat and not casein, such as fermentative production of NOCs, could be contributing to DNA damage. It has not been possible to examine for NOCs in this study because of sampling limitations, but future investigations are warranted to further elucidate their influence on colorectal oncogenesis.

Resistant starch supplementation ameliorated DNA adduct formation. This, in turn, was associated with a reduction in rates of TUNEL-identified apoptosis. This apoptosis might be triggered, at least in part, by the amount of unrepaired DNA damage or errors and so a reduction might be explained by a reduction in adduct formation. The correlation data suggest that DNA adduct attenuation by resistant starch was directly related to reduced protein fermentation, increased SCFA production, and lower fecal pH. This reduction in DNA adducts may equate to protection against CRC risk, as epidemiologic evidence supports the protective effect of resistant starch (48).

An observation of increasing DNA adducts from the bottom to the top of the crypt was seen in all experimental dietary groups. It is likely that more adducts observed toward the top of the crypt may relate to age of the cells

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and hence exposure time to the diet. As cells proliferate and differentiate toward the lumen, exposure time to the contents of the lumen is longer than newly generated cells, thereby these cells have more time to accumulate adducts but at low levels such that repair or apoptotic deletion fails to remove them. It is also possible that it reflects greater exposure through the effects of a concentration gradient. It is thought that the reactive species leading to adduct formation are generated in the digesta so that exposure would be greatest at the cells closest to the luminal surface. The relevance of DNA adduct formation at the luminal surface of cells and cancer risk remains unclear, as it is possible that DNA in cells not destined for cellular division can act as a sink for adduct formation and afford protection to cells actively proliferating.

In conclusion, this is the first study to show that high protein diets increase promutagenic adducts in the colon (O^6MeG) and it is the type rather than the amount of dietary protein that is important. The protein fermentation product *p*-cresol correlated with adduct formation. Delivering extra fermentable carbohydrate to the colon via resistant starch supplementation results in a switch from fermentation of protein to fermentation of carbohydrate and a reduction in adduct formation, suggesting that dietary resistant starch reduces the mutagenic effect of red meat in the diet.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

J. Winter, G.P. Young, M.A. Conlon, A.R. Bird, D.L. Topping, and R.K. Le Leu were involved in the design of the study. J. Winter and R.K. Le Leu were responsible for execution of the experimental work and data collection. L. Nyskohus was responsible for development of the adduct methodology. R.K. Le Leu and J. Winter for the data analysis and writing of the manuscript.

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