Accumulation of pro-mutagenic DNA adducts in the mouse distal colon after consumption of heme does not induce colonic neoplasms in the western diet model of spontaneous colorectal cancer

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Abbreviations: colorectal cancer (CRC), resistant starch (RS), 8-hydroxy-2 deoxyguanosine (8-oxo), O^6-Methyl-2-deoxyguanosine (O^6MeG), aberrant crypt foci (ACF), N-nitroso compound (NOC), short chain fatty acids (SCFA), high amylose maize starch (HAMS), azoxymethane (AOM)

Keywords: heme, red meat, resistant starch, 8-hydroxy-2 deoxyguanosine, O^6-Methyl-2-deoxyguanosine,

Abstract

Red meat is considered a risk factor for colorectal cancer (CRC). Heme is considered to promote colonic hyperproliferation and cell damage. Resistant starch (RS) is a food that ferments in the colon with studies demonstrating protective effects against CRC. Utilising the western diet model of spontaneous CRC we determined if feeding heme (as hemin chloride) equivalent to a high red meat diet would increase colonic DNA adducts and CRC and whether RS could abrogate such effects. Four groups of mice: Control, Heme, RS and Heme+ RS were fed diets for 1 or 18 months. Colons were analysed for apoptosis, proliferation, DNA adducts '8-hydroxy-2 deoxyguanosine' (8-oxo) and 'O^6-Methyl-2-deoxyguanosine' (O^{6}MeG) and neoplasms. In the short term, heme increased cell proliferation ($p<0.05$). Changes from 1 to 18 months showed increased cell proliferation ($p<0.01$) and 8-oxo adducts ($p<0.05$) in all groups, but only heme-fed mice showed reduced apoptosis ($p<0.01$) and increased O^{6}MeG adducts ($p<0.01$). The incidence of colon neoplasms was not different between any interventions. We identified heme to increase proliferation in the short term, inhibit apoptosis over the long term and increase O^{6}MeG adducts in the colon over time although these changes did not affect colonic neoplasms within this mouse model.

Introduction

Colorectal cancer (CRC) is a major burden on public health in developed countries with high incidence and mortality rates globally [1]. Although the development of CRC can be attributed partly to familial inherent mutations, a major driving force behind CRC formation is related to lifestyle factors, in particular dietary choices [2]. The consumption of red meat has

been identified as a risk factor for developing CRC by the World Cancer Research Fund [3]. However, the mechanisms by which red meat might initiate oncogenesis in the colon are not clearly known.

Increased DNA alkyl adducts in the colon via excess endogenous N-nitrosation is one mechanism that is thought to play a role in colorectal oncogenesis, although little is known about DNA adduct formation in the colon in response to red meat consumption [4] [5]. Our recent studies have shown that red meat can enhance the production of the pro-mutagenic adduct O^6-Methyl-2-deoxyguanosine (O^6MeG) after feeding of high red meat diets to mice, without chemical carcinogenic intervention [4]. The O^6MeG adduct is a known mutagenic lesion in both animals and humans resulting from exposure to alkylating agents and is repaired by the enzyme O^6-methylguanine-DNA methyltransferase. Persistence of unrepaired O^6MeG adduct changes the chemical bonds in the DNA backbone and thus the DNA polymerase reads the G as an A after one round of replication. If this mismatch is not repaired, further changes in the DNA backbone lead to the polymerase incorporating a T instead of a C, therefore a GC:AT transition mutation arises following a second round of replication [6]. This transition mutation has been identified in several genes including the *K-ras* gene, where mutation of this gene is a known mechanism of human oncogene activation in CRC [6].

The DNA oxidative adduct 8-hydroxy-2 deoxyguanosine (8-oxo) has been implicated in development of cancer for several years [7]. 8-oxo is a pro-mutagenic adduct that if not repaired or removed from the cell can incorporate base insertions into the DNA, with G → T tranversions being the most abundant [8]. The major repair pathway for this DNA adduct is via base excision repair, with the DNA glycosylase enzyme OGG1 having an affinity for excising the 8-oxo adduct from the DNA [9]. Patients with CRC and inflammatory bowel disease present with higher levels of 8-oxo measured in plasma [10, 11] and CRC patients show higher levels of 8-oxo DNA adducts measured in colon tissue compared to patients

without CRC [12, 13]. 8-oxo adducts are clearly linked with carcinogenesis although the significance of the 8-oxo adduct and consumption of red meat or heme in CRC has not been studied extensively.

Heme iron in red meat has been implicated in development of CRC in humans [14]. There are many different proteins regulating heme iron absorption in the small intestine and although it is readily bio-available there is a limitation to its absorption, therefore it is easily able to reach the colon [15, 16]. Various animal studies have shown that diets with heme concentrations above average human consumption levels (0.25-1.5µmol/g) [17] contribute to cell toxicity, hyper-proliferation, reduced apoptosis and increased rates of CRC [16-22]. A diet incorporating low levels of heme (at 0.25µmol/g) has shown a reduction in total number of aberrant crypt foci (ACF) per colon, although the ACF size was larger with heme diets compared to a control diet [18]. The intake of heme from a 30% red meat diet as beef would equate to an average concentration of 0.14µmol/g [19], indicating that 0.25µmol/g of heme more closely resembles that of a high red meat intake in humans. Besides causing damage to the colon cells directly, heme-iron from red meat also triggers the formation of potentially damaging DNA alkylating N-nitroso compounds (NOC) either via increasing bacterial nitrate reductase activity or via reacting with nitrite or nitric oxide in the lumen [20]. In humans, consumption of high levels of red meat has been directly related to increased alkyl adducts in the colon, and this was correlated to increased faecal NOC output [21]. There are strong indications that heme contributes to development of CRC. However to date, there have been no rodent studies conducted examining a heme diet at levels relevant to human consumption to identify its long term effects on colorectal cancer risk without utilising chemical carcinogens to induce cancer to date utilising solely,. However, to directly assess the carcinogenic potential of dietary heme without utilising chemical carcinogens or other spontaneous models huge numbers of animals per group are required to detect any possible effect on carcinogenicity.

On the opposing side, a dietary component that has been implicated in protection against CRC is resistant starch (RS), the component of starch that is undigested in the small intestine and fermented rapidly in the colon. It is thought that the preferential fermentation of carbohydrate over protein, when RS is incorporated into high protein diets, leads to a reduction in DNA changes that might initiate CRC [4, 22]. This fermentation increases the production of short chain fatty acids (SCFA) [23], in particular butyrate, which is important for large bowel function and is the major energy source for colon epithelial cells. RS has also been shown to reduce production of toxic protein fermentation products [4, 24]. *In vivo* animal models demonstrate the ability of butyrate to increase the acute apoptotic response to a chemical carcinogen and reduce cellular proliferation to allow for repair or removal of highly damaged cells [25]. Studying the effect of RS in human subjects is complex due to varying RS sources and maintaining adequate dosing, thus it is difficult to directly pinpoint the protective effect RS has against CRC in humans [23, 24] [30-33]. Daily intake of fibre sources in the above human studies were mostly over 30g, but actual RS intake would differ due to varying RS content in different RS food sources. This suggests that the dose of RS might not have been achieved in some of these studies to have a direct effect on the colon and higher doses of RS should be aimed for.

As heme is considered to be oncogenic, either directly or indirectly, and RS has the capacity to reduce oncogenic DNA lesions in the colon, we investigated whether feeding heme (at human-relevant amounts) to mice as part of the western diet model of spontaneous colorectal cancer for short or long periods of time could induce pro-oncogenic adducts or colonic neoplasms, and if epithelial events relevant to formation of DNA lesions were affected. We also aimed to find out if adding RS at levels appropriate for human dietary intake could manipulate these parameters.

Materials and methods

Animals and Diets

A total of 225 eight week old wild type c57bl/J 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 (ethics approval number 809/12). Animals were randomly placed four mice per cage and divided into dietary groups under controlled conditions of 22 ± 2ºC (SD), 80 ± 10% humidity and 12h light/dark cycle. Animals had free access to food and water and were weighed once weekly throughout the study period. 60 mice consumed diets for four weeks (short term) and 160 mice were on diets for 18 months (long term).

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 western diet model of spontaneous colorectal cancer created by Newmark et al [26, 27] (Table 1). Our experimental diets were a modification of the AIN76 diet to give 15% total protein using casein (w/w) and 15% protein with 10% high amylose maize starch (HAMS). HAMS was supplied by the National Starch and Chemical Company, Bridgewater, NJ, and contains approximately 50% RS [28], therefore a total of 5% RS was added to the diet. The amount of heme used in this study is at a final concentration of 0.2μmol/g (supplied as hemin chloride, Sigma catalogue # 51280). This heme concentration resembles the heme content from a high red meat diet [4, 19]. Final diet preparations were placed into air sealed containers, stored at 4°C with fresh food in the mouse cages replaced daily.

Faecal Analysis

After three weeks (short term) or six months (long term) on experimental diets fresh faecal samples were collected from each mouse. For measurement of faecal pH, faeces were placed into three times the weight-to-volume of cold saline, vortexed until emulsified and pH measured using a glass embodied electrode (Eutech Instruments). Another fresh faecal sample was placed into three times the weight-to-volume of a SCFA/phenols internal standard (800ml Milli Q water, 120μl heptanoic acid, 50mg of o-Cresol, pH 7.0). Duplicate

100µl aliquots were used to measure SCFAs (butyrate, acetate and propionate) and phenols and *p*-cresols. For SCFAs, 100µl was injected into an Agilent Technologies 6890N Network Gas Chromatograph System fitted with a Zebron ZB-FFAP column (0.53 mm × 30 mm) and measured as described previously [29]. For phenols and *p*-cresol, another 100µl of distillate was injected into a Shimadzu LC-10AD HPLC machine with RF-10AXL fluorescence detector set at excitation 284nm, emission 310nm and measured as described previously [30].

Tissue Sample collection

All colon samples were fixed with 10% buffered formalin solution containing 3.6% formaldehyde for 24 hours then transferred to 70% ethanol for processing. On completion of short term experimental diets 1cm of distal colon was used for analysis. After the long term feeding experiment the entire colon was cut longitudinally for analysis of tumours under a dissecting microscope before a 1cm distal colon segment was taken for analysis and all tumours were collected and processed for H&E staining for histological confirmation. Distal colonic tissue and tumours were processed through gradient alcohols and xylene, embedded in paraffin wax before sectioning on a microtome.

Cell Proliferation and Apoptosis measurement

Proliferative activity of distal colonic epithelial cells was measured using an antibody specific for the nuclear proliferating antigen ki-67 (rat-anti-mouse clone TEC-3, Dako, USA) in combination with an immunohistochemistry detection method in paraffin embedded sections, as used previously [31]. Slides were visualised under light microscopy by brown nuclear staining and proliferation was assessed as the ki-67 positive cells per crypt column length. Apoptosis was measured using a terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay kit (Trevigen, USA) in paraffin embedded sections, following the manufactures instructions. Slides were visualized under light microscopy 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.

DNA Adduct Quantification

DNA alkylation was quantified using an antibody specific for the DNA adduct O^6-Methyl-2-deoxyguanosine (O^6MeG, Clone EM 2-3,Squarix Biotechnology, Germany) and the level of oxidative adducts was measured using an antibody specific for the DNA adduct 8-hydroxy-2 deoxyguanosine (8-oxo, Trevigen, USA) combined with a mouse-on-mouse polymer horse radish peroxidase (Poly-HRP) detection system (Covance Laboratories) on paraffin-embedded distal colon sections. The primary antibody concentration for O^6MeG was 1:1000 (in PBS) and for 8-oxo the primary antibody was applied at 1:2000 (in PBS). Immunohistochemical staining procedures and computer image analysis of stained slides were carried out as previously described [4]. Total sum of adduct formation was calculated along the crypt length 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 standard error. Tumour outcomes are expressed as incidence (proportion of mice that develop neoplasia). General linear model univariate analysis was used to determine the main effect of RS addition and heme on all outcomes measured. Independent sample t-tests were performed on each variable to determine the difference between short term and long term feeding of each diet on DNA markers and cell kinetics. Colon tumours were analysed by Crosstabs Fishers Exact test. The null hypothesis was rejected at the 0.05 level for all statistical tests carried out using SPSS version 19 (IBM).

Results

Body Weight and Faecal analysis

In the short term, no significant differences in body weight (g) were observed after consuming the experimental diets for four weeks although after long term feeding mice consuming dietary heme had significantly reduced body weights compared to mice on control diet (p<0.01) (Table 2). After both short and long term feeding, addition of RS to the diet significantly lowered faecal pH compared to those diets without RS (p<0.0001) and markedly increased faecal total SCFAs concentration (p<0.0001) including acetate (p<0.006), propionate (p<0.0001) and butyrate (p<0.0001) (Table 2). RS was also able to reduce concentration of faecal *p*-cresol (p<0.0001) and phenol (p<0.0001) in the short term (Table 2). Heme addition had no significant effect on faecal fermentation of SCFAs, phenol or p-cresols compared to the control after long term feeding (Table 3). Although when heme was combined with RS in the long term experiment there were significantly reduced concentrations of acetate (p<0.001), propionate (p< 0.05), butyrate (p<0.05) and total SCFAs (p<0.01) (Table 3) compared to the Control + RS diet. After long term feeding diets containing RS showed significantly reduced fermentation of phenols but not p-cresols compared to diets without RS (p<0.05).

Short-term effects of heme and RS

In the short term, heme at 0.2µmol/g in the diet increased cell proliferation (Figure 1B) as measured by positive ki-67 cells in the colon compared to diets without heme (p<0.05). RS significantly increased crypt height in the colon compared to diets without RS (p<0.05) but it did not increase the proportion of ki-67-positive cells (Figure 2B and 2C). There were no significant effects of heme or RS on apoptotic cell counts in colonic epithelial cells (Figure 1A and 2A). There were no significant differences after four weeks of feeding in either the O^6MeG adduct (Figure 1D and 2D) or the 8-oxo adduct (Figure 1E and 2E) between heme and control diets or after the addition of RS to the diet.

Long-term effects of heme and RS

After 18 months on experimental diets, those mice fed heme showed no changes in proliferation, crypt height, apoptosis or DNA adduct levels compared to mice fed a heme-free diet (Figure 1A-1E). Diets containing RS increased colonic crypt height (p<0.01) as well as increasing cell proliferation (p<0.01) compared to diets without RS (Figure 2B and 2C). However, RS did not affect apoptosis or DNA adducts (Figure 2C, 2D and 2E). Although it appears heme lowered neoplasm incidence in the colon, there were no statistically significant differences in colon neoplasm incidence for any of the dietary interventions (Table 4).

Diet-related changes over time

To ascertain if diets led to changes over time we compared short and long term feeding of heme and RS (Figure 3). Dietary heme significantly lowered rates of apoptosis in older mice compared to their younger counterparts (Figure 3A, p<0.01). Cell proliferation increased and crypt height decreased with age (Figure 3B and 3C) for all dietary treatments (p<0.01). Diets that contained heme (p<0.01) or heme with RS (p<0.001) showed a significant increase in O^6MeG DNA adduct accumulation over time; whereas diets without heme (Control and Control + RS) showed no significant difference between the two time points (Figure 3D). 8-oxo adducts in the colon accumulated over time (Figure 3E) with all 4 dietary interventions (Control: p<0.05, Control + RS: p<0.001, Heme: p<0.01 and Heme + RS: P<0.000001).

Discussion

We have identified dietary heme at a human relevant amount to increase proliferation in colon cells of mice in the short term, inhibit colonic apoptosis over the long term and contribute to accumulation of O^6MeG pro-mutagenic adducts in the colon over time. Interestingly, these adverse changes invoked by this level of dietary heme did not increase

rates of colon neoplasms within the western diet model of spontaneous colorectal cancer in the mouse.

There is evidence that increased heme intake from red meat will catalyse the production of more N-nitroso Compounds (NOC) in the colonic lumen, leading to increased risk in developing colon cancer [20]. Heme-induced increases in NOC can lead to increased alkylating adducts in the colon, as NOC are potent initiators of such alkylating adducts [21]. We observed an accumulation of O^6MeG adducts over time with heme consumption, however this did not accelerate colorectal oncogenesis under the conditions of the experiment. It may be possible that as mice age they become more susceptible to accumulation of DNA adducts in the presence of heme, possibly due to the cells being more inefficient at repairing such adducts with increasing age. Measurement of DNA repair of oncogenic adducts might be warranted in future studies to determine if the aging process effects DNA repair mechanisms and therefore accumulation of DNA lesions with consumption of high red meat diet**s.**

Heme alone may catalyse the production of NOC's but red meat and particularly processed meat [32] contain NOCs that might further contribute to alkylating DNA lesions of the colon. This could be one reason why heme (at the studied dose) alone did not influence rates of CRC. Clearly, the alkyl adducts generated were not sufficient to initiate or promote CRC. Whether this was due to failure to reach a sufficient number or whether it was in the context of a low-risk setting for CRC, given that no other mutagen was given or that there was no high risk factor present, we cannot be certain. This might be further explored by feeding higher amounts of heme but such would be above what is reasonably encountered in the human diet. It is likely that NOC generation in mice fed the heme diets would be minimal as NO and NO_2 (required for NOC generation) were not added to the diet or water, and rodent saliva does not recycle NO_2 as human saliva does [33]. Due to sampling limitations,

NOCs were not measured in the current study, but this would be warranted in future studies examining dietary heme as a cancer causing agent. It might also be that the duration of the current study may need to be extended beyond 18 months to see if there is further accumulation of O^6MeGadducts and whether this increases risk for CRC. The diets we based our experiments on were adapted from Newmark et al [26, 27] where end-points of 18 and 24 months saw significant increases in CRC compared to normal diets. However, due to a significant weight loss in heme-fed mice (table 3), as well as some early losses of mice in all dietary interventions resulting from the low nutritional quality of the westernised diet, 18 months was used as the cut-off point in our study to avoid further loss of mice.

A high level of dietary heme has been previously shown by other researchers to increase cell proliferation and decrease apoptosis in the colon of rats, possibly as a result of increased oxidative stress and a loss of cell feedback between damaged cells and proliferating cells [34]. We also observed increased cell proliferative activity in the short term and a reduction of apoptosis over the long term in the current study, but this did not equate to a higher CRC risk. More recently, Ijssennagger et al [35] showed 0.2μmol of heme was sufficient enough to induce acute oxidative stress and delayed cytotoxic stress in the lumen of mice after only 2-14 days of heme consumption, with a dramatic increase in proliferating colon cells. In a study where heme was used at a similar concentration of 0.25μmol/g ACF size increased, however, there was a statistically significant reduction in number of azoxymethane (AOM)-induced ACFs per colon (after 100 days) compared to the control diet (92 ± 16 compared to 125 ± 25) [18]. There are some distinct differences with this study including different base diets used and the treatment with AOM to drive the CRC pathway, whereas we used only heme as the potential carcinogenic agent. This indicates that heme may increase the risk of developing CRC at higher doses but not at lower doses more relevant to human consumption over a longer period of time, and could explain why we saw no significant effect on CRC in the current study. According to two studies [17] [19] the level of heme-iron in cooked red meat as beef is on average 2.63mg/100g of meat. This equates

to an average concentration of heme in a 30% red meat diet to be 0.14μmol/g [19]. Most studies have used much higher doses, up to 10 times this amount of heme, ranging from 0.25μmol/g up to 1.5μmol/g of diet [18, 34, 36], which is quite unrealistic in the context of human consumption. The concentration of heme in our study at 0.2μmol is very similar to that of the high red meat diet of 30%, representing a maximum intake of heme achieved in humans. Although heme may disrupt homeostasis of the cell by increasing proliferation and decreasing apoptosis, this had no influence on initiation or promotion of colorectal cancer. The effect of heme in the form of hemin on colon carcinogenesis remains questionable, with levels of heme present in a relatively normal diet not affecting CRC risk in the western diet model of spontaneous colorectal cancer. However, more studies in other animal models which also include a more detailed dose evaluation are required to confirm heme's effect on colorectal carcinogenesis.

Our findings on the effects of dietary RS support previous literature that this food source acts to reduce toxic protein fermentation and acts as a promoter of SCFA production, particularly the anti-cancer compound butyrate. There is a firm belief that the preferential fermentation of RS in the colon over protein leads to increased production of chemo-protective compounds and reduction of potentially toxic compounds in the lumen [24]. Rodent models consistently show fermentation of RS and production of SCFAs is linked to decreased rates of DNA lesions in colon cells, ACFs and tumours in chemical carcinogenic models of colorectal cancer [22, 25, 37]. We have shown previously that RS is able to reduce DNA adducts induced by high red meat diets, but not high casein diets, without the use of chemical carcinogens, and this was related to increased SCFA and decreased protein fermentation [4]. The levels of heme used here did not induce detectable changes in DNA adducts between individual diets (both alkylating and oxidative) in the short term to detect a protective effect of RS despite increased SCFA production. Although Control + RS diet appeared to increase O^6MeG adducts over time, there was a large variation between the mice and therefore this did not reach statistical significance. Also, over the long term,

addition of RS was not able to reduce the observed increase of adducts induced by heme and one reason for this could be the decrease in SCFA production by RS in the presence of heme after long periods. It is likely that the bacterial population in the colon has shifted with consumption of heme over longer periods, favouring a move away from beneficial bacteria fermentation provided by an RS diet. Previous studies have confirmed that consumption of dietary heme does create shifts in populations of bacteria in the colonic lumen [38]. Due to the small faecal sample size of the mice it was not possible to measure bacterial profile but this would be warranted in future studies to identify the interaction of heme with other dietary agents.

We have identified dietary heme as an agent that can increase DNA pro-mutagenic adducts in the colon of mice, increase cell proliferation and reduce apoptosis rates in the colon. We also confirm that RS can promote beneficial bacterial fermentation and reduce toxic protein fermentation in the colon of rodents over short periods, but SCFA production was not sustained over longer periods in combination with heme intake. All these changes observed had no influence on CRC risk when heme was studied at a human relevant amount with the western diet model of spontaneous colorectal cancer. The level of heme in a typical western diet may not be sufficient to initiate or promote CRC alone. This suggests that heme may act with other components of red and processed meat, such as NOCs, contributing to DNA damaging events in the colon that might lead to colorectal carcinogenesis.

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Table 1: Composition of Experimental Diets in g/100g

Ingredient	Control	Control + RS	Heme	Heme + RS
Casein	17.6	17.6	17.6	17.6
Corn starch	10	-	10	-
Hi-Maize™	-	10	-	10
Sucrose	45.48	45.48	45.48	45.48
Sunflower Seed oil†	16.8	16.8	16.8	16.8
Lard	3.2	3.2	3.2	3.2
α-cellulose	2	2	2	2
L-cysteine	0.3	0.3	0.3	0.3
Choline	0.12	0.12	0.12	0.12
Minerals*	3.5	3.5	3.5	3.5
Vitamins*	1	1	1	1
DL-Methionine	0.15	0.15	0.15	0.15
Hemin#	-	-	0.01304	0.01304

* 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 mg/g, and vitamin D3 at 0.11 IU/g.

† Sunflower seed oil contained no added antioxidants.

Hemin is at 0.2µmol/g of total diet.

Table 2: Short Term Study Measures of Body Weight and Fermentation[1]

Variable	Unit	Experimental Diet Groups			
		Control (n=12)	Control + RS (n=12)	Heme (n=12)	Heme + RS (n=12)
Body Weight	g	28.5 ± 0.4	28.4 ± 0.5	29.3 ± 0.9	28.6 ± 0.5
Fecal pH	-	7.8 ± 0.2	7.1 ± 0.2[a]	7.6 ± 0.1	7.0 ± 0.1[a]
Total SCFAs	µmol/g	45.6 ±11.3	109.9 ± 10.4[a]	55.6 ± 12.2	117.3 ± 16.9[a]
Butyrate	µmol/g	1.8 ± 0.8	19.2 ± 3.2[a]	4.9 ± 0.9	20.4 ± 3.1[a]
Acetate	µmol/g	36.1 ± 8.7	61.2 ± 6.6[a]	42.0 ± 9.8	68.1 ± 10.3[a]
Propionate	µmol/g	7.6 ± 1.9	29.6 ± 2.5[a]	8.7 ± 1.8	28.8 ± 4.1[a]
Phenol	µg/g	7.2 ± 1.6	0.8 ± 0.6[a]	7.3 ± 1.7	0.2 ± 0.1[a]
***p*-cresol**	µg/g	21.7 ± 2.5	11.8 ± 1.6[a]	21.5 ± 3.3	9.3 ± 1.1[a]

[1] Data represented as Mean ± SE

[a] Control + RS and Heme + RS is significantly different to Control and Heme

Table 3: Long Term Study Measures of Body Weight and Fermentation[1]

Variable	Unit	Experimental Diet Groups			
		Control (n=40)	Control + RS (n=40)	Heme (n=40)	Heme + RS (n=40)
Body Weight	g	58.3 ± 2.1	52.6 ± 1.8	48.0 ± 2.1[a]	51.7 ± 1.4
Fecal pH	-	7.7 ± 0.2	7.1 ± 0.2[a]	7.5 ± 0.1[d]	6.9 ± 0.1[a,c]
Total SCFAs	µmol/g	25.3 ± 3.8[b]	52.4 ± 5.1[a]	22.3 ± 3.1[b]	19.0 ± 2.6[b]
Butyrate	µmol/g	4.9 ±1.5[b]	18.8 ± 1.9[a]	6.8 ± 1.3[b]	6.2 ± 0.7[b]
Acetate	µmol/g	17.7 ± 2.8	15.1 ± 1.6	8.4 ± 1.0[a]	7.1 ± 1.3[a,b]
Propionate	µmol/g	2.8 ± 0.3[b]	14.8 ± 1.6[a]	4.5 ± 0.7[b]	3.5 ± 0.5[b]
Phenol	µg/g	2.0 ± 0.7	0.9 ± 0.1	1.8 ± 0.2	1.2 ± 0.4
p-cresol	µg/g	7.5 ± 2.1	11.1 ± 1.7	7.8 ± 0.7	6.7 ± 1.1

[1] Data represented as Mean ± SE

[a] significantly different to Control

[b] significantly different to Control + RS

[c] significantly different to Heme

[d] significantly different to Heme + RS

Table 4: Main effects of Dietary Interventions on Colon Neoplasm Incidence

Heme Effect	Incidence (%)	RS Effect	Incidence (%)
No Heme	4.45	No RS	3.35
Heme	0	RS	1.1
p value*	0.134	p value*	0.523

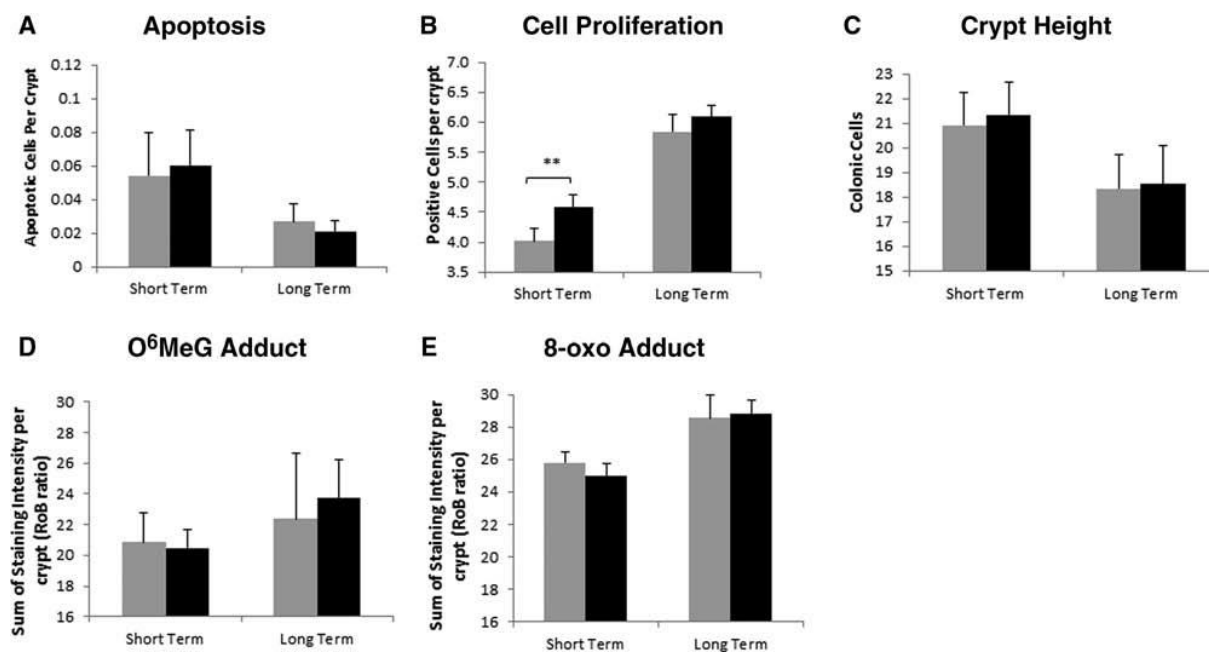* Cross tabulation performed for colon cancer using Fishers exact test.

Figure Legends

Figure 1: Effect of dietary heme on apoptosis (A), cell proliferation (B), colonic crypt height (C), O^6MeG adduct (D) and 8-oxo adduct (E) load in the distal colon after short term and long term experiments. Diets without heme represented by grey bars, diets with heme represented by black bars. Data are represented as means ± SE. **$p<0.01$

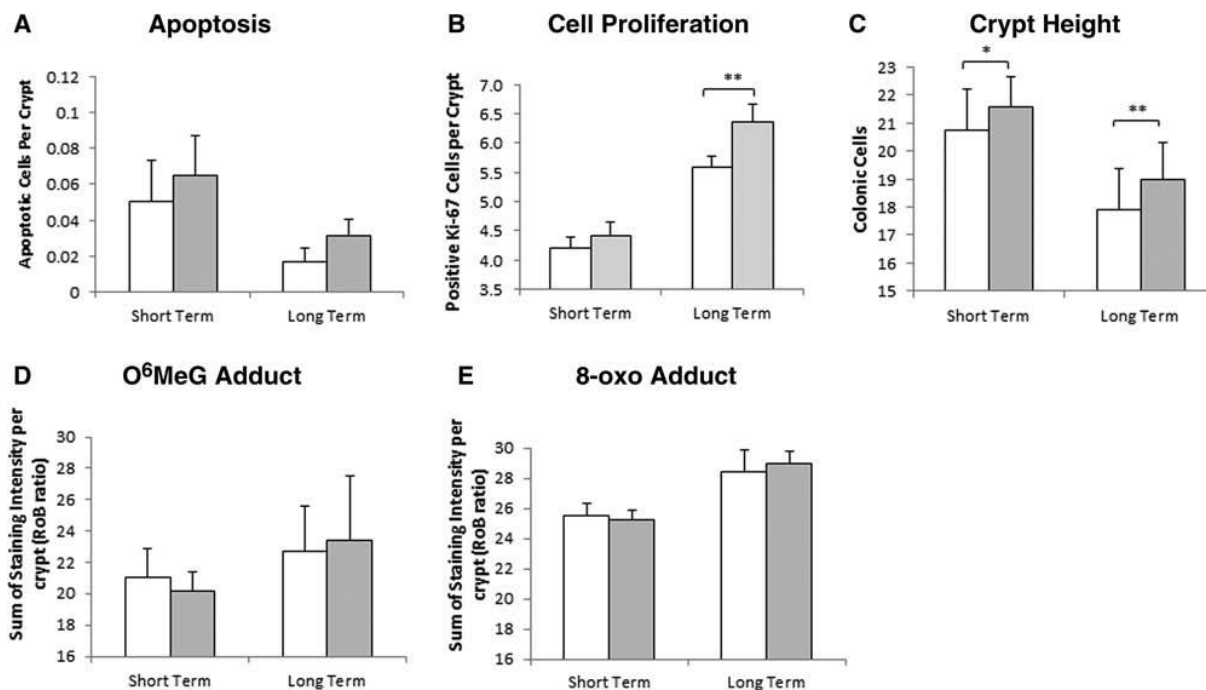**Figure 2:** Effect of dietary RS on apoptosis (A), cell proliferation (B), colonic crypt height (C), O^{6}MeG adduct (D) and 8-oxo adduct (E) in the distal colon after short term and long term experiments. Diets without RS represented as white bars, diets with RS represented as grey bars. Data are represented as means ± SE. *p<0.05, **p<0.01
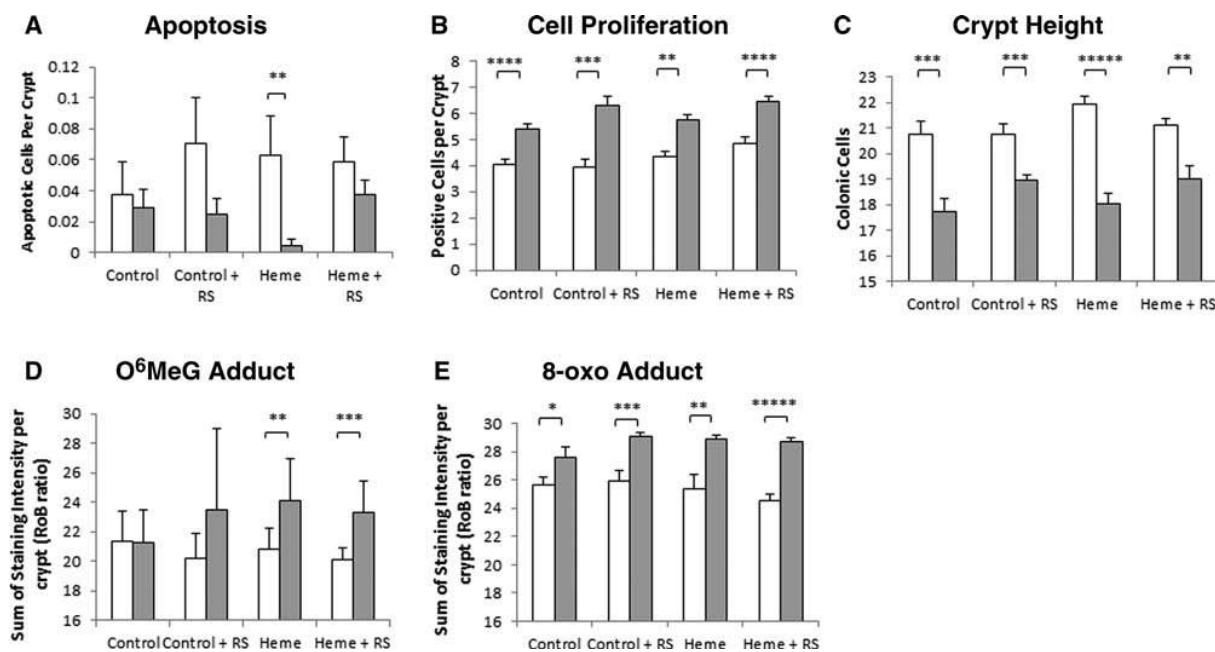
Figure 3: Changes in the distal colon over time from 4 weeks (white bars) to 18 months (grey bars) for apoptosis (A), cell proliferation (B), colonic crypt height (C) O^6MeG adduct (D) and 8-oxo adduct (E). Data are represented by means ± SE. *p<0.05, **p<0.01, ***p<0.001, ****p<0.000001, *****p<0.000001