Effect of dietary resistant starch and protein on colonic fermentation and intestinal tumourigenesis in rats

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Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in affluent countries and is a leading cause of cancer-related mortality in the USA and Australia (1,2). Accumulating evidence from epidemiological and experimental studies suggest that diet is an important environmental factor in the aetiology of CRC. The typical Western diet contains high concentrations of proteins including red meat, high-fat dairy products and processed foods. These dietary components may increase the risk of CRC through various mechanisms, including oxidative stress, DNA damage, inflammation and the production of carcinogenic compounds (3,4). On the contrary, there is low consumption of fruits, vegetables, starches and whole grains that have been suggested to be protective against CRC (5–7).

Resistant starch (RS) is a component of dietary starch that is not absorbed in the small intestine of healthy individuals and thus reaches the colon undigested, similar to dietary fibre. RS can be classified into four main types, of which the first three may occur in a typical human diet (8). RS1 includes physically entrapped starch within whole plant cells and food matrices (e.g. coarsely milled grain). RS2 consists of native starch granules that are highly resistant to digestion by α-amylases (e.g. green banana, high amylose maize starch). RS3 comprises retrograded starches, formed when starchy foods are cooked and cooled. RS4 comprises chemically modified starches (e.g. esterified starches) where the modification interferes with the amylolytic activity of digestive enzymes (RS5 are used in the food industry). RS is a potential source of fermentable substrate for anaerobic bacteria resulting in the production of gases (CO2, CH4, and H2) and short-chain fatty acids (SCFAs) (9). The SCFA butyrate has generated the most interest as it may be protective against CRC (10,11). Butyrate is the primary energy source for colonic epithelium (12), and it also inhibits the growth of cancer cells in vitro and forces a more normal differentiated phenotype (10). In addition, it is a potent pro-apoptotic agent (13) that might aid removal of cells with damaged DNA. Colonic production of butyrate by fermentation is associated with reduced tumour mass in an animal model, provided that fermentation is active in the distal colon (14). Consumption of a high-RS diet has been shown to increase fermentation products (particularly butyrate) and this has been suggested as a means of protection in South African blacks (15). Furthermore, population studies suggest that CRC risk can be lowered by increased RS consumption possibly through increased butyrate production (7). Fermentation may be important in prevention of CRC via other effects including altering gut microbiota toward a more beneficial composition microflora (16,17).

Previous studies in our laboratory have shown that RS can facilitate the acute apoptotic response to a genotoxic carcinogen in the rat colon (18). RS might regulate mutational load in the colon and eliminate DNA-damaged cells that might otherwise progress to malignancy, thereby exerting a protective effect at the early stages in the onset of cancer (19). RS was also shown to influence fermentation parameters such as lowering pH, increasing SCFA production including butyrate, while also stimulating desirable bacteria within the colon (20). RS may also protect through broader mechanisms associated with fibre such as faecal bulking, decreased transit time and altered pH (9). All these effects might mediate protection.

Protein and amino acids also pass into the colon where they are available for bacterial fermentation. Around 13 g of protein material may enter the colon each day (21,22). Dietary sources make up at least 50% of this protein material; however, the amount may vary due to protein intake and the physical form of the food (21,23). Other sources of protein in the colon include bacterial secretions such as enzymes, sloughed-off epithelial cells, bacterial lysis products and mucins (22). Evidence from ileostomy studies suggests that it is the amount of protein in the diet rather than its source that determines the amount reaching the colon (23). Protein fermentation products that may be produced in the colon include branched-chain fatty acids (BCFAs) (isobutyric and isovaleric acids) (24) and potentially toxic substances such as ammonia, amines, N-nitroso compounds, phenols, thiols and indoles (25). Production of the latter are increased after supplementary dietary protein in humans (26,27). Experimental evidence is accumulating from animal models and in vitro data, which shows that dietary proteins can influence cancer expression (28,29). Recently, animal studies have shown that increased dietary protein consumption can cause increased colonic DNA damage, as measured by the comet assay, and thinning of the colonic mucus barrier, whereas with inclusion of RS into the diet, these damaging effects are attenuated (30,31). Furthermore, SCFA production, particularly butyrate, may be enhanced if indigestible carbohydrates like RS are combined with dietary protein sources like rice, soy or potato (32).

This study was designed to investigate the interaction of RS2 and/or undigested dietary protein on colonic luminal environment and the incidence and number of small intestinal neoplasms including: no added RS or PP, 10% high amylose maize starch (source of HAS), high amylose cornstarch; PP, digestion-resistant potato protein; RS, resistant starch; SCFA, short-chain fatty acid.
relationship of this to intestinal cancer formation in Sprague–Dawley rats. Potato protein was chosen as the dietary protein source because of its digestibility so as to ensure that more protein reaches the colon. In addition, we determined the effects of these diets on bacterial β-glucuronidase activity. β-Glucuronidase is an enzyme that is believed to be largely responsible for hydrolysis of glucuronide conjugates in the intestine and is thought to play an important role in the generation of toxic and carcinogenic substances (33).

Materials and methods

**Animals and diets**

A total of 120 male Sprague–Dawley rats, 5 weeks of age, were obtained from the Animal Resource Centre, Perth, Western Australia. Animals were divided randomly into four experimental groups and housed three per plastic cage in an animal holding room under controlled conditions of 22 ± 2°C (SD), 80 ± 10% humidity and 12 h light–dark cycle. Animals were given free access to water and weighed weekly throughout the study.

The diets were modified forms of the AIN-76a standard for purified diets for rats and mice (34). Each group of animals was fed an experimental diet based on the control diet. The control diet contained 200 g casein, 461.5 g cornstarch, 109.5 g sugar and 180 g sunflower oil/kg diet. Choline, methionine, minerals and vitamins were added as described previously (20). The experimental diets varied in protein type and density between treatment groups. The first group ‘control’ consumed a diet containing no added fibre or RS. The second group ‘RS’ had a diet that contained 100 g/kg raw high amylase cornstarch (HAS), the third group ‘PP’ had a diet that contained potato protein isolate 150 g/kg diet. The fourth group’s diet ‘PP + RS’ contained 150 g/kg potato protein and 100 g/kg raw HAS. High amylase cornstarch (Hi-maize® 958), an RS, was used as the source of RS and was supplied by the National Starch and Chemical Company, Bridgewater, NJ. High amylase cornstarch was added to the diets so as to displace an equal amount of digestible cornstarch. Potato protein was supplied by Hokuren Co. (Hokkaido, Japan) and was prepared from potato juice by steam coagulation at pH 5.0–5.5 as described previously (32). The chemical and amino acid compositions of this protein have been reported previously (35). The protein content of the protein isolate was 60.7% (as is basis) as measured by the Kjeldahl’s method using 6.25 as the conversion factor. The true digestibility of potato protein was determined by the method of Njaa (36) under isonitrogenous condition in rats by using the following equation: true digestibility = [nitrogen intake – (faecal nitrogen excretion – faecal nitrogen excretion in the protein-free diet)/nitrogen intake × 100] and was found to be 84.0 ± 0.7% (SD) and was significantly lower compared with casein 95.0 ± 0.3% (SD). The potato protein was added to the PP diets at the expense of an equal amount of casein.

**Experimental procedure**

After 4 weeks on experimental diets, each rat received s.c. injections of azoxymethane (15 mg/kg body wt) (Sigma Chemical Co., St Louis, MO) once weekly for 2 weeks and then maintained on their dietary regimen until killed at 30 weeks. The rats in each group were weighed once weekly. In the fourth week of the experiment, 15 rats from each treatment group were placed in metabolic cages to measure faecal output, food intake and collect urine. Fresh faecal samples were collected from each rat and diluted in three volumes of internal standard solution (heptanoic acid, 1.68 mmol/l) and stored at −20°C for later analysis of SCFA concentrations. Table II summarizes the SCFA concentrations in the caecum, proximal and distal colonic contents and faeces of rats fed the different experimental diets.

**Intestinal tumours**

Using a light microscope (magnification ×40), the small intestine and colon were scored for tumour number and location by an independent observer who was unaware of dietary treatment as described previously (37). Tumours were removed and embedded in paraffin (5 μm) for histopathological analysis. All tumours were examined histologically and evaluated by an independent observer based on the criteria of Pozharisski (38). Adenoma was characterized by expansion of the mucosal layer, reduction in goblet cell number, cellular dysplasia and moderate loss of mucosal architecture by glandular growth and lack of invasion through the basement membrane. Adenocarcinoma was identified from the following characteristics: typical cytological change, prominent cellular atypia, loss of cell polarity, marked distortion of glandular architecture and invasion (38).

**Statistical analysis**

Data are presented as the mean ± SEM for each treatment group. The effect of RS and protein on tumour data and fermentation outcomes were analyzed by creating dummy variables for RS, PP and their interaction, and entering them into an appropriate generalized linear model. If the interaction term was not statistically significant (P ≤ 0.05), the regression model was rerun omitting the interaction effect to assess the significance of the two main factors. A Gaussian generalized linear model was used for continuous data, Poisson for neoplasm development and spectrophotometric assay. The results are expressed as phe- nolphthalein released (mg) per caecal content (g) per hour.

**Results**

**Body weights, food intake and faecal output**

Table I summarizes weight gain, food intake and faecal output. The PP group gained less weight (g/30 weeks) over the duration of the study (P = 0.001). Daily food intake tended to be lower with PP (P = 0.002). Faecal output was increased by feeding RS and PP (P < 0.001).

**Effect of the diets on colonic carbohydrate fermentation**

Table II summarizes the SCFA concentrations in the caecum, proximal and distal colonic contents and faeces of rats fed the different experimental diets. The SCFA concentrations in the caecum were approximately twice those of the faeces. Cacal total SCFA (P < 0.001) and acetate concentration (P = 0.001) were elevated with RS. RS and indigestible dietary protein showed an interactive effect (RS × PP, P = 0.001) on cecal propionate and butyrate, with the highest butyrate concentration achieved when combining RS with PP (i.e. PP + RS).

The SCFA concentrations in the proximal colonic content were slightly lower than that in the caecum and displayed similar relative differences to those within the caecum. In the distal colon, RS significantly elevated total acetate and butyrate concentrations (P < 0.001). An interaction effect (RS × PP) was observed on propionate concentration in the distal colon (P = 0.029). In the faeces, both total SCFA (P = 0.001) and butyrate (P = 0.002) concentrations were significantly elevated by RS supplementation. Interactions between RS and PP were observed for both acetate (P = 0.024) and propionate (P = 0.01) concentrations in the faeces.

**Effect of diets on protein fermentation products**

BCFA concentrations were consistently higher with indigestible protein than with other diets (Table III). An interaction between RS and
PP (RS × PP) was observed with caecal BCFA (P = 0.049). Proximal BCFAs were significantly lower with RS (P < 0.001) and significantly higher with PP (P < 0.001). Distal (P = 0.004) and faecal (P = 0.014) BCFAs were elevated with PP.

Indigestible protein (PP) significantly increased the concentration of urinary p-cresol (P < 0.001) whereas RS had the opposite effect (P < 0.001). An interaction between PP and RS was observed for urinary phenol, with the RS interacting with the PP to lower the concentration (P < 0.001).

**Effect of diets on caecal β-glucuronidase activity**

In the caecum, β-glucuronidase activity was significantly reduced with RS (P < 0.001) (Figure 1).

**Colonic neoplasms.** The effects of diet on incidence (number of rats with neoplasms) and the number and type of azoxymethane-induced neoplasms in the colon are shown in Table IV. RS protected against adenocarcinoma formation by reducing the incidence of rats affected whether or not PP was present in the diet (P = 0.038). PP alone was not protective. A similar trend that did not reach significance was seen for the effect of RS on total number of adenocarcinomas.

**Intestinal neoplasms.** The effects of diet on incidence (number of rats with neoplasms) and the number and type of azoxymethane-induced neoplasms in the small intestine and small intestine combined with colon are shown in Table V. RS feeding significantly lowered both the incidence and numbers of total neoplasms (P = 0.01) and of
Incidence (%)b

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| Neoplasms (number)c
| All neoplasms   |              |         |    |    |        |    |    |         |
| Adenomas        |              |         |    |    |        |    |    |         |
| Adenocarcinomas |              |         |    |    |        |    |    |         |

*Mean (SEM), n = 30. High amylase cornstarch used as source of RS; digestion-resistant potato protein (PP).  
+Effects tested with log binomial generalized linear model.  
+Effects tested with Poisson generalized linear model.

Fig. 1. Effect of resistant starch (RS) and digestion-resistant potato protein (PP) on caecal β-glucuronidase activity in rats.

Table IV. Effect of RS and potato protein on measures of azoxymethane-induced colonic neoplasmsa,b

Adenocarcinomas (P = 0.008) in the small intestine. In contrast, PP feeding promoted the incidence of total neoplasms (P = 0.004) and adenocarcinoma incidence (P = 0.003) in the small intestine.

When the neoplasms of both the small intestine and colon were combined, RS protected against overall adenocarcinoma incidence (P = 0.002) and total number of intestinal adenocarcinomas (P = 0.001).

Discussion

Our results have demonstrated that there are many interactions between starch and protein that have major effects on luminal environment with also significant implications for oncogenesis.

RS supplementation altered the luminal environment throughout the large bowel and in faeces. SCFA concentrations in the caecum and colon were all elevated with RS feeding. RS feeding also lowered the production of potentially harmful by-products of protein fermentation such as phenols and cresols. Furthermore, the activity of bacterial β-glucuronidase was diminished. β-Glucuronidase is an enzyme that is believed to be largely responsible for hydrolysis of glucuronide conjugates in the intestine and is thought to play an important role in the generation of toxic and carcinogenic substances (33). These effects might also contribute to protection.

Although RS had a favourable effect on the luminal environment, feeding the indigestible potato protein showed that increased colonic protein might create effects that could be detrimental. Results showed that incorporation of indigestible protein significantly increased markers of protein fermentation evident by higher BCFAs in the caecum and colon and elevated levels of the putrefactive catabolites phenol and p-cresol. These putrefactive protein catabolites have been postulated to have negative influences on gut health (26). It is possible that the increased production of the putrefactive catabolites may be an indirect effect of dietary proteins effect on the activity of undesirable bacteria populations (such as sulphate-reducing bacteria, *Escherichia coli*, Clostridia and *Bacteroides*). The typical Western diet of humans contains high concentrations of protein with as much as 13 g of protein being able to escape digestion and enter the colon on a daily basis (22). Increased delivery of protein to the colon results in greater amino acid fermentation with production of potentially toxic substances such as ammonia, amines, N-nitroso compounds, phenols, thiols and indoles (25). We chose potato protein in the current study because it has a lower digestibility than that of casein and so serves to test what happens when extra protein escapes digestion in the small intestine and reaches the colon (32).

As a diet, protein and carbohydrate are consumed together. In the present study, we have shown that the carbohydrate RS can modulate the fermentation effects of dietary protein. It has been shown previously that indigestible potato protein promotes caecal butyrate production (32), an effect that may be explained by the indigestible protein altering the ratio of carbohydrate:nitrogen in the colon, thereby further sustaining fermentation. It was suggested from this study that indigestible proteins have an important physiologic role through interaction with RS in providing butyrate to colonic tissues. However, in the present study, although a significant interaction between RS and indigestible potato protein was seen in the caecum with increased caecal butyrate levels, this effect was not sustained through the colon. Any perceived benefit of enhanced caecal butyrate levels may be counteracted by increased production of potentially toxic fermentation substances throughout the gastrointestinal tract. The fermentation of protein by the colonic microflora was substantially modified by the supplementation of RS, as evidenced by a decrease in the concentration of putrefactive compounds and BCFA. This phenomenon has been observed previously in animal studies (30) as well as human intervention studies (42,43). Previous studies by Toden et al. (30) also demonstrated that RS may attenuate colonic DNA damage induced by consumption of a high dietary protein diet.

In parallel with its effects on starch and protein fermentation, RS feeding protected against intestinal tumourigenesis, including the development of colonic adenocarcinoma. This suggests that a favourable
shift in the luminal environment achieved through RS feeding contributes to protection against intestinal tumourigenesis. In the distal colon, which is the site where tumours predominate (44), butyrate concentration was markedly increased with the RS supplementation. Butyrate is thought probably to promote colonic health and protect against CRC. Colonic production of butyrate by fermentation has previously been associated with reduced tumour mass provided that fermentation is active in distal colon (14) and is often hypothesized as a protective factor against CRC (7,45). Previous studies from our laboratory have shown that fermentable substrates such as RS or wheat bran can increase apoptosis in response to a genotoxic carcinogen, and furthermore, this appears to be dependant on the concentration of the carcinogen (18,46). Increased apoptosis during initiation events might enhance the elimination of mutated cells that might otherwise progress to malignancy (19). Such an effect might be a mechanism by which RS acts to protect against CRC. Other factors such as a decrease in harmful protein catabolites (phenols and cresols) and bacterial enzymes may also contribute to RS protective effects. Several studies have shown that carcinogen-induced tumour incidence is associated with a modification of bacterial β-glucuronidase activity. An increase in bacterial β-glucuronidase activity is often associated with a higher tumour risk (47) whereas a decrease in β-glucuronidase activity has been associated with a reduction in tumour incidence (48).

The effect of RS supplementation in previous animal studies on tumourigenesis is limited and conflicting (49–53) and is summarized in Young et al. (49). More recently, hydrothermally treated RS3 protected against dimethylhydrazine-induced colon carcinogenesis (54). There are several explanations for the varying results between the reported studies as follows: the different carcinogen protocols (dose and duration), differences in starch type, feeding regimens and the comparative control diet. Some of these will alter luminal conditions known to have a major influence on colonic oncogenesis (9,55). RS can be classified into four main types based upon structural considerations and bacterial degradation (8), each with different characteristics and different fermentation patterns (56) that may lead to different effects on luminal environment. As a consequence, different types of RS should not be considered equivalent.

Increased delivery of protein to the colon promoted intestinal tumourigenesis, although the effect was obvious in small intestine and not colon. Consumption of high protein diets in rats has previously been shown to increase the genetic damage in the colon as measured by the comet assay (30). Why indigestible protein promotes tumourigenesis in small intestine but not colon in the present study is not entirely clear and needs more investigation. Although harmful fermentative products are produced in the colon, they might act systemically on small intestine thereby increasing tumourigenesis. When RS was combined with indigestible protein, no enhancement of small intestinal tumourigenesis was observed and this might be due to the local effect of higher concentrations of butyrate, as indigestible protein promotes fermentation of carbohydrate to butyrate. Enhanced colonic production of butyrate raises not only the portal level of butyrate but also the arterial level (57), suggesting that butyrate potentially may have effects on cells not in direct proximity to the gut (58). Certainly, RS decreased protein fermentation products and decreased the promotional effect of indigestible protein on intestinal tumourigenesis.

Changes in the luminal environment in response to delivery of starch and/or protein to the colon are sizeable and complex—they also correlate with risk for cancer. RS favourably influences the colonic luminal environment through enhanced butyrate production and reduction in harmful bacterial enzymes and products of protein fermentation. These conditions are associated with reduced risk of tumourigenesis in the colon and small intestine. Increased protein delivery to the colon increases the accumulation of potentially harmful by-products of aromatic amino acids. This set of conditions is associated with increased intestinal tumourigenesis but adding RS reduces such accumulation and protects against protein-enhanced tumourigenesis.

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Resistant starch protects against colorectal tumourigenesis


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