THE INTERACTION BETWEEN DIETARY PROTEINS AND RESISTANT STARCH ON LARGE BOWEL HEALTH

A thesis submitted to the University of Adelaide for the degree of Doctor of Philosophy

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Shusuke Toden

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ABSTRACT

A review of the literature revealed that diet plays an important role in serious human non-infectious large bowel diseases including cancer and inflammatory bowel diseases. Dietary protein (especially as red and processed meats) has been implicated as a positive risk factor for colorectal cancer while starch which is not digested in the small intestine (resistant starch, RS) appears to be protective. The series of experiments described in this thesis were aimed to determine the effects of dietary proteins and RS on indices of colon health in an animal model, the laboratory rat. Genetic damage is a prerequisite for carcinogenesis and this was assessed by a specific assay (the comet assay) which gives a measure of DNA strand breaks. Loss of mucus barrier function is thought to contribute to inflammatory bowel disease by permitting bacterial translocation and this was measured optically using a microscope micrometer. Other biomarkers were measured as described below. There were four major experiments.

1. **Effects of dietary red meat and casein on colonic DNA damage and interaction with resistant starch**

Previous studies had shown that higher dietary protein (as casein) induced genetic damage in rat colonocytes and that RS (fed as a high amylose maize starch) was protective. This study was aimed at establishing whether a high protein diet fed as cooked red meat had similar effects and whether RS was protective. Rats were fed diets containing either 15 % or 25% casein or 25% barbecued lean red beef, each with or without 48% high amylose maize starch (as a source of RS) for 4 weeks. As expected, high dietary casein caused a 2-fold increase in colonic single-strand DNA breaks compared with a low casein diet and

reduced the thickness of the colonic mucus layer by 41%. High levels of cooked meat caused 26% more DNA damage than the high casein diet but reduced mucus thickness to a similar degree as casein. Addition of RS to the diet abolished the increase in DNA damage and the loss of colonic mucus thickness induced by either high protein diet. It is thought that RS promotes large bowel health through the SCFA produced by the large bowel bacteria. One acid in particular (butyrate) has been associated particularly with promotion of normal large bowel function and protection against disease. In keeping with this hypothesis, caecal and faecal short chain fatty acid pools (including those of butyrate) were increased by inclusion of RS in the diet. DNA damage is an early step in the initiation of cancer and these findings agree with the population data which suggest that total dietary protein and red meat promote risk of colorectal cancer. However, inclusion of resistant starch in the diet could significantly reduce that risk.

2. Differential effects of dietary whey, soy and casein on colonic DNA damage and interaction with resistant starch

The preceding experiments showed that high levels of animal-derived proteins increased colonocyte genetic damage and loss of the mucus barrier in rats. This second experiment was designed to determine whether diets high in different types of dairy protein (casein or whey) or a plant protein isolate (soy) had similar adverse effects on colonic DNA and mucus barrier function and whether inclusion of RS in the diet was protective. Adult male Sprague Dawley rats were fed a diet containing 15 % or 25 % casein, whey or soy protein, each with or without 48 % high amylose maize starch for 4 weeks. In confirmation of the earlier studies, higher levels of dietary casein increased colonocyte DNA damage significantly. However, whey did not increase genetic damage. Colonic DNA damage

was highest for soy when fed at both 15% and 25% protein in the absence of RS. Inclusion of RS in the diet attenuated colonocyte DNA damage due to higher dietary protein in all three groups. The colonic mucus barrier was thinner in rats fed higher dietary protein but the effect was reversed by feeding RS. Caecal total SCFA and butyrate pools were low in rats fed the digestible starch and were higher in rats fed RS. However, there was no relationship between caecal or faecal SCFA and genetic damage or mucus thickness. Caecal and colonic tissue weight and colon length were higher in rats fed RS, consistent with greater SCFA supply. These data confirm that higher dietary protein of animal (casein) or plant (soy) origin increases genetic damage and loss of the mucus barrier indicating that this is an effect of protein and not its source. These findings accord with the epidemiological data which link dietary protein to greater risk of colorectal cancer and inflammatory bowel disease. However, the data show also that dietary proteins differ in their specific actions on genetic damage and mucus thickness. Further, the data from the feeding of whey suggest that not all proteins are equivalent in their capacity to provoke adverse changes in colonic integrity. While the data show that RS raised large bowel and faecal SCFA, they indicate their levels were not related directly to these biomarkers.

3. Dose response effects of resistant starch on protein induced colonic DNA damage

The accumulated data linking greater protein intakes to adverse changes in the colon were obtained at dietary levels which were not unreasonable in terms of animal or human consumption. However, the dietary level of RS which were fed were relatively high (48% by weight) so this study was conducted to determine its effectiveness at lower levels of

dietary inclusion. It was also important to ascertain whether there was a dose-response relationship between RS intake and the observed effects. One of the mechanisms proposed for the induction of colorectal cancer by high dietary protein intakes is oxidative damage to DNA. In this experiment, this was done by assaying with endonuclease III. Adult male rats were fed a diet containing 25% casein with 0%, 10%, 20%, 30% or 40% high amylose maize starch for 4 weeks. As in the preceding studies comet tail moment was greatest and the mucus barrier thinnest in rats fed 0% RS. DNA damage was reduced and the mucus barrier thickened in a logarithmic dose-dependent manner by RS. There was no significant difference between dietary groups associated with oxidative DNA damage as measured by endonuclease III. Caecal and faecal short chain fatty acid (SCFA) pools rose with the increased level of dietary RS. DNA damage of colonocytes correlated negatively with caecal SCFA but the strongest correlation was with caecal butyrate, which is consistent with the proposed role of this SCFA in promoting a normal cell phenotype. The data show that RS prevents protein induced colonic DNA damage in a dose-dependent manner. Inclusion of 10% high amylose maize starch was found to be sufficient to oppose colonocyte DNA damage, and to increase caecal and faecal SCFA pools. Intakes of this order are not unreasonable in terms of human consumption of RS.

4. Dose response effects of red and white meat on colonic DNA damage and interaction with resistant starch

The accumulated evidence from large prospective human studies links diet to colorectal cancer risk strongly. The evidence from the animal studies described in this thesis that dietary protein induces colonocyte genetic damage supports a role for high protein intakes in increasing risk. Recently, several large epidemiological studies and a meta-analysis of

prospective studies have found that consumption of dietary red or processed meats, but not white (poultry) meat, is associated with increased risk of colorectal cancer. This is consistent with the data from the preceding studies that specific proteins affected colonic integrity differentially. A large prospective European study (European Prospective Investigation into Cancer and Nutrition) has reported that dietary fibre was protective. The findings reported in this thesis that RS opposes the effects of high dietary protein accord with that conclusion. This study aimed to compare the effects of cooked red (beef) or white (chicken) meat on DNA damage and mucus barrier thickness in rats. The study was designed to determine whether the relationship between the intakes of these meats was dose-dependent. Double-strand DNA breaks are thought to relate more closely to carcinogenesis than single-strand breaks so both were measured. Adult male Sprague-Dawley rats were fed a diet containing 15%, 25% or 35% cooked beef or cooked chicken each with or without 20% high amylose maize starch for four weeks. Both red and white meat increased colonic DNA damage dose-dependently. However, both single and double strand breaks were significantly greater when the rats were fed the red meat diets compared to those fed the white meat. Colonocyte DNA damage was reduced by the consumption of RS while large bowel SCFA were increased. The findings of this study are consistent with the epidemiological data which show that red meat consumption is associated with greater risk of colorectal cancer but that white meat is not.

Summary

The data reported in this thesis support the findings of prospective population studies that high dietary protein, red meat in particular, appears to be harmful to the health of the large bowel. However, the data demonstrate also that different protein types have differential

effects on the integrity of the colonocyte DNA. Furthermore, the addition of RS to the diet protects against protein-induced colonic DNA damage and maintenance of the colonic mucus barrier, apparently through increased SCFA production by colonic fermentation. The results of these experiments indicate a strong potential for RS to be effective in maintenance of large bowel integrity in the face of high dietary protein.

PUBLICATIONS ARISING FROM THESIS

Toden, S., Bird, A.R., Topping, D.L. & Conlon, M.A. High Red Meat Diets Induce Greater Numbers of Colonic DNA Double-Strand Breaks than White Meat In Rats: Attenuation by High Amylose Maize Starch. Carcinogenesis 2007 ***In Press***

Toden, S., Bird, A.R., Topping, D.L. & Conlon, M.A. Dose-dependent reduction of dietary protein-induced colonocyte DNA damage by resistant starch in rats is more highly correlated with levels of caecal butyrate than other short chain fatty acids. Cancer Biology and Therapy Feb; 26(2):253-258, 2007

Toden, S., Bird, A.R., Topping, D.L. & Conlon, M.A. Differential Effects of Dietary Whey, Casein and Soy on Colonic DNA Damage in Rats. British Journal of Nutrition 97(3):535-543, 2007

Toden, S., Bird, A.R., Topping, D.L. & Conlon, M.A. Resistant starch prevents colonic DNA damage induced by high dietary cooked red meat or casein in rats. Cancer Biology and Therapy Mar;5(3):267-72, 2006

Toden, S., Bird, A. R., Topping, D. L., and Conlon, M. A. Differential effects of dietary whey and casein on colonic DNA damage. Aust. J. Dairy Technol. 60, 146-148 2005

PRESENTATIONS

Conference Abstracts

Conlon, M.A., **Toden, S.**, Bird, A.R., Topping, D.L. Resistant starch and colonic mucosal integrity. Asia Pacific Journal of Clinical Nutrition **15** *Suppl:* S45, 2007

Toden, S., Bird, A. R., Topping, D. L., and Conlon, M. A. Resistant starch protects against colonic DNA damage induced by dietary whey, soy and casein in rats Dietary Fibre 2006 Multifunctional Complex of Components 160-161, 2006

Topping, D.L., Bird, A.R., Regina, A., **Toden, S.**, Rahman, S., Keogh, J., Conlon, M.A., Morell, M.K. New cereal foods for improved human health, American Association of Cereal Chemsists Symposium, San Francisco, USA, 2006

Topping, D.L., Bird, A.R., Clarke, J., Conlon, M.A., Keogh ,J., Li, J.L., Morell, M., Regina, A., Rahman, S., **Toden, S.** Engineering Starches for Foods to Improve Human Health, Asia Pacific Congress of Chemical Engineers, Kuala Lumpur, Malaysia, 2006

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Topping, D. L., **Toden, S.**, Bird, A. R. and Conlon, M. A. Resistant starch and health, University of Illinois,Chicago IL, USA, 2005

Topping, D.L, Bird, A.R., **Toden, S.**, Conlon M.A., Noakes M., Morell M., Mann G., and Li, Z.L. Interaction between dietary proteins and resistant starch Making Fiber Irresistible: Resistant Starch is a Natural Seminar, Chicago IL, USA, 2005

Topping, D.L., Bird, A.R., **Toden, S**., Conlon M.A., Noakes M., King R., Morell M., Mann G., and Li, Z.L. Resistant Starch: Strategies to Increase Intakes for Health Benefit. National Starch Food Innovation, Health Professionals Symposia, Sydney and Melbourne, 2005

Topping, D., Bird, A.R., **Toden, S.**, Conlon M.A., Noakes M., King R., Morell M., Mann G., and Li, Z.L. Resistant starch as a contributor to the health benefits of whole grains. Whole Grains Summit, Minneapolis, MN, USA, 2005

ABBREVIATIONS

ACF	aberrant crypt foci
APC	adenomatous polyposis coli
AIN	American Institute of Nutrition
ANOVA	analysis of variance
BaP	benzo(a)pyrene
cm	centi-meter
CRC	colorectal cancer
ºC	degrees, Celsius
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMH	dimethylhydrazine
DTT	dithiothreitol
DSB	double-strand break
FAP	familiar polyposis
g	grams
GSH	glutathione
GST	glutathione S transferase
h	hours
HBSS	Hanks' balanced salt solution
HCA	Heterocyclic amines
HAMS	High amylose maize starch,
HDAC	histone deacetylase

IBD	Inflammatory bowel disease
M	molar
µg	micrograms
µl	milligrams
mm	millimetres
min	minutes
NOC	N-nitrosocompounds
NSP	non-starch polysaccharides
PBS	phosphate buffered saline
PAH	Poly aromatic hydrocarbons
pH	potential of hydrogen
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
RS	Resistant starch,
SCFA	short chain fatty acid
SEM	standard error of the mean
SSB	single-strand break
TCF	transcription factor
TBS	tris buffered saline
wk	weeks

CHAPTER 1: RESEARCH BACKGROUND

The main goal of the work described in this thesis is to determine the effects of high protein diets and RS on large bowel health. This was achieved by comparing the differential effects of several types of dietary protein on colonic DNA damage as an indicator of potential colorectal cancer risk in rats. The potential protective effects of RS on colorectal cancer risk were examined through changes in genetic damage. The effects of the two dietary treatments (RS and dietary protein) on colonic barrier function were examined through determining changes in mucus barrier thickness. The following research background is intended to give the reader an understanding of the current state of knowledge on the actions of different types of protein on the risk of large bowel diseases. Furthermore, this review details information relating to the protective effects of dietary fibre, especially RS, and its potential protective mechanisms against large bowel diseases.

1.1 Diet and Bowel Health

It is well established that there is a very strong link between diet and large bowel health. The principal function of the large bowel is to salvage water from digesta passing from the small intestine. While the absorption of nutrients is largely completed when digesta reaches the terminal ileum, there is a potential for significant dehydration without this recovery (1-3). The period of exposure of the colon wall to digesta is relatively long while this process is completed, increasing the risk of potential damage by carcinogens and toxins.

1.1.1 Bowel Diseases

There are several non-infectious large bowel diseases which have significant effects on human health. These are inflammatory bowel diseases (IBD; Crohn's disease (CD) and ulcerative colitis (UC)) and colorectal cancer (CRC). Although IBD and CRC are different diseases, it is known that patients with prolonged IBD are at increased risk for developing colon cancer (4-7). UC patients have at least 3 times higher colon cancer risk than in the general population and about 5 % of people with UC develop colon cancer (5, 6). CD patients, with at least 30% of the colon involved in disease, may have an increased risk of colorectal dysplasia and cancer (6). The risk of cancer increases with the severity of the damage to the colon and the duration of the disease. Although it is difficult to associate specific dietary components to IBD risk, studies indicate that CD is increasing in countries where a more Western diet (high in refined foods) is displacing more "traditional" diets (high in unrefined food and dietary fibre) (8).

1.1.1.1 Inflammatory Bowel Diseases

IBD are more common in Europe and North America where they affect both males and females equally (9). The disease affects people of all ages, but is more common in those aged 15 to 40 years. However, many recent reports have highlighted the rising prevalence of IBD in Asia (10-12). It is estimated that 61,000 Australians have IBD; approximately 28,000 have CD and 33,000 have UC (13). IBD is a group of inflammatory conditions of the large intestine, and in some cases the small intestine. IBD is thought to result from inappropriate and ongoing activation of the mucosal immune system driven by the presence of normal luminal flora and this response is facilitated by defects in both epithelial barrier functions and the mucosal immune system (14). Minor forms of IBD also

include: collagenous colitis, lymphocytic colitis, ischaemic colitis, diversion colitis, Behcet's syndrome, infective colitis and indeterminate colitis.

The major difference between UC and CD is that, the former only affects the inner lining of the bowel wall, whereas in CD the inflammation is of the full thickness of the bowel wall and can affect the whole gastrointestinal tract. The most common symptoms of UC include: abdominal pain, blood, mucus or pus in the stool, diarrhoea, fatigue and tiredness, weight loss and loss of appetite (15). Although the cause of UC is still unknown, causative factors suggested include: genetic predisposition, infectious agents, defects in immune system and environmental factors.

As noted, CD involves any part of gastrointestinal tract, but most frequently involves the distal small bowel and colon. Inflammation can vary greatly from a small ulcer over a lymphoid follicle to an extensive chromic inflammation. This inflammation is transmural, and can result in strictures, micro-perforations, and fistulae. CD is often associated with autoimmune disorders outside the bowel, such as aphthous stomatitis and rheumatoid arthritis (16).

1.2 Colorectal Cancer

Western countries including Europe, America and Australia have the highest incidence of colon cancer and account for nearly two-thirds of the approximately one million cases that occur worldwide each year (17). However, many Asian countries including, Japan, China and South Korea have experienced a two to four fold increase in the incidence of CRC in recent decades (18, 19). CRC is the second most common cause of cancer-related death in both men and women in western countries (20). In Australia,

CRC was the second leading type of cancer death in 2004, second only to skin cancer (21, 22).

Genetic factors are thought to be important in CRC development in individuals (23). Among first-degree relatives of colon cancer patients, the lifetime risk of developing CRC is 18%, a 3-fold increase over the general population (21). However, 80% of CRC occur sporadically in patients with no family history of CRC, indicating environment has a large bearing on development of CRC. Diet is considered to be the main environmental factor, with approximately 70% of CRC attributed to an inappropriate diet (23, 24). DNA damage is a prerequisite for cancer induction and it is established that specific food ingredients or their metabolic by-products are genotoxic (25-27). Other environmental factors include viral infections, chemical carcinogens, and radiation. Viral infections and radiation exposure are rare causes of CRC, while dietary carcinogens are much more important because of their direct exposure to the gut.

1.2.1 Pathogenesis of Colorectal Cancer

Cancer is a complex multi-step process and thought to be caused by an accumulation of multiple genetic mutations resulting in a transformed phenotype (28). Genomic instabilities caused through mutation or lack of DNA damage repair can lead to cancer formation (29, 30). There are two major possible processes that may be responsible for inducing genetic mutations in colonocytes: 1) the direct action of a mutagen causing genetic damage or 2) increased proliferation causing an increased risk of errors in DNA repair. A cell accumulating these genetic mutations will likely to undergo full malignant transformation (31). This concept, known as the “multi-step model of carcinogenesis” is

described in detail below (**Fig. 1.1**).

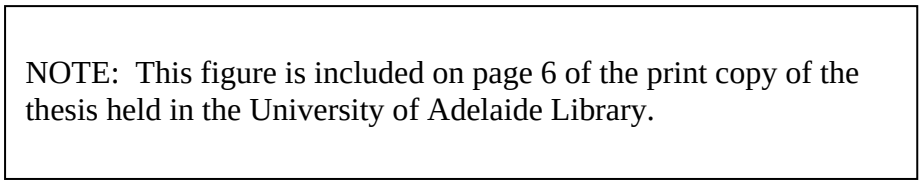

Figure 1.1 Multiple steps of colorectal carcinogenesis. Significant genes involved in gene regulation in carcinogenesis include adenomatous polyposis coli (APC), K-ras and p53 (Modified from Sharma *et al.* 2001)

Initiation, the first stage of colorectal carcinogenesis, is often associated with mutation in the adenomatous polyposis coli (APC)/β-catenin pathway or mismatch repair genes. Both genes play a pivotal role in the regulation of mucosal proliferation and are classic tumour suppressor genes. Mutations in the APC gene on chromosome 5q21 locus are found in 60 to 80% of sporadic CRC and adenomas (32). In sporadic CRC, mutation in

APC initiate the majority of the tumours (33). Mutations in the APC gene are responsible for the disease familiar polyposis (FAP), where patients develop numerous benign tumours of the colon. Some of these tumours will progress to cancer if not removed surgically. APC is known to regulate β-catenin-TCF or Wnt signalling. The APC protein interacts in a complex with β-catenin, glycogen synthase kinase 3β and axin to regulate the levels of β-catenin by targeting β-catenin for degradation by the ubiquitination-proteosome pathway (34). In the absence of functional APC, β-catenin levels rise, enabling it to form an active complex with the transcriptional factor TCF-4 (35). The β-catenin-TCF-4 transcription complex targets c-myc (36), which activates the transcription of the cyclin-dependent kinase, cdk 4 and consequently increases cell proliferation (37).

A number of different agents have been found to cause genetic damage to colonocytes. Some of the known agents include dietary contaminants, chemical mutagens, pathogenic bacteria and viruses. Mutations may not necessarily lead to the development of cancer as apoptosis, programmed cell death, can remove cells with genetic mutations (38). In addition, there are many non-genotoxic agents that are associated with increased risk of CRC. These agents include hormones, drugs, physical or mechanical trauma and other chronic irritants (39). Increased cell proliferation is a common histological change induced by these agents which can potentially lead to carcinogenesis.

The K-ras oncogene is thought to be involved in transition from intermediate adenomas to carcinomas in sporadic CRC (33). The K-ras gene product, located on the inner plasma membrane, is involved in the transduction of mitogenic signals. The ras protein is activated transiently as a response to extra-cellular signals that stimulates cell surface receptors (40).

The final stage of carcinogenesis is progression. It involves additional growth of

the adenoma and invasion of the basement membrane. Loss of p53, a tumour suppression gene, is the major genetic change associated with progression of tumour growth to carcinoma. The p53 gene is known to arrest cell cycle progression, increase apoptosis and allow the DNA to be repaired. In cancer cells with mutated p53, cell proliferation is no longer controllable, resulting in inefficient DNA repair and the emergence of genetically unstable cells (41).

CRC can be viewed as a process of polyp formation. Although benign polyps do not invade nearby tissue or spread to other part of the large intestine, they can become malignant over time. The figure below shows the development of CRC through a series of histological stages from adenocarcinoma to carcinoma (42) (**Fig. 1.2**).

NOTE: This figure is included on page 8 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.2 Multi-stage model of rat colon cancer. (Based on Nakagama et al 2005)

1.2.2 Genomic Instability, DNA Damage and Cancer

Recent studies have suggested that the link between DNA damage and early stage cancer is strong (43-46). DNA damage occurs all the time and the majority of time this damage is repaired by DNA repair mechanisms. Therefore, DNA repair is an essential process for cell survival because it protects the genome from potentially harmful mutations. However, accumulation of this damage can lead to activation of proto-oncogenes and inactivation of tumour suppressor genes, which may lead ultimately to cancer formation. Tumourigenesis driven by genetic damage is fuelled by DNA damage and errors made by the DNA machinery (47).

Genomic instability can take two main forms; microsatellite instability and chromosomal instability (48). Microsatellites are stretches of DNA in which a short motif is repeated several times. The most common microsatellite in the humans is a dinucleotide repeat of CA which occurs frequently across the genome (49). Microsatellite instability occurs when a germline microsatellites allele has gained or lost repeated units and thus undergone a somatic change in length (49). Chromosomal instability, or gross rearrangement of chromosomes, occurs when the cells show aneuploidy, a chromosomal state in which abnormal numbers of chromosome sets exist within the nucleus with chromosomal breaks and other defects (50). Furthermore, point mutations, changes in a single base pair, can affect a range of genes with strong contributory links to CRC. As noted previously, these include the oncogene K-ras, and tumour suppressor gene p53. Point mutations can arise through various mechanisms. Spontaneous mutation may occur due to the instability of purine and pyrimidine bases or defects in mismatch repair (51). Common chromosomal aberrations include the loss or gain of whole chromosomes or

chromosome fragments and the amplification of chromosome segments (52). Loss of large regions of chromosomes can lead to the inactivation of tumour suppressor genes, whereas amplification of chromosomal regions might promote tumourigenesis by activation of proto-oncogenes (53).

1.2.2.1 DNA Adducts

DNA adduct formation is another mechanism of mutagenesis. A DNA adduct is the covalent linking of an abnormal radical to DNA with the potential for cancer formation (main carcinogens include: N-nitrosamines, aflatoxins, aromatic amines, and polycyclic aromatic hydrocarbons) (54, 55). The misreplication of DNA adducts can lead to malignancy. Alkylating or oxidative agents cause the majority of documented adducts. Recently, there have been speculations that these adducts are linked to greater CRC risk (56, 57). O^6-methylguanine DNA adducts are formed between nitrosated glycine derivatives and DNA (56, 58). Glycine, a common dietary amino acid, opens the possibility that its nitroso products could be major alkalylating agents in the human gastrointestinal tract (59, 60). Furthermore, recent *in vitro* studies have demonstrated that formation of O^6-methylguanine DNA adducts lead to mutations in p53 similar to those observed in human gastrointestinal tract tumours (61). A study in human volunteers has shown that dietary red meat consumption led to significantly higher O^6-methylguanine DNA adducts than white meat in colonocytes (57). In addition, a study in rodents treated with a carcinogen (AOM) showed that a combination of fish oil and pectin (a highly fermentable fibre) reduced the amount of O^6-methylguanine DNA adducts (62). Collectively, these data suggest that adduct formation is a potential mechanism for

carcinogenesis which can be modulated by diet to increase or decrease risk.

1.2.2.2 DNA damage (single and double-strand DNA breaks)

Chromosome breaks are a documented measure of DNA damage with single-strand DNA breaks (SSB) being one of those measured most frequently. SSB arise constantly from DNA base damage and must be repaired to maintain genomic stability. If the repair process is hindered, this can lead to double-strand breaks during DNA replication and can result in chromosome instability and cell death. DNA double-strand breaks (DSB) are considered to be one of the most dangerous forms of cellular genomic damage. DSB differ from other types of DNA lesion in that both strands of the double helix are damaged which prevent the use of the complementary DNA strands as a template for repair. The accumulation of DNA damage can lead to cancer or serious impairment of cellular functions **(Fig. 1.3)**. DSB result from exogenous agents such as ionising radiation and certain chemotherapeutic drugs, endogenously generated reactive oxygen species, mechanical stress on the chromosome and specialized recombination reactions (52, 63).

To counteract DNA damage, all organisms possess DNA repair mechanisms that correct specific types of lesions. Extensive damage can lead to cell death. Cells can enter 'replicative death', a state of irreversible growth arrest, or trigger apoptosis (52). Other protective mechanisms include cell-cycle check points and DNA-damage repair. An epidemiologic study showed consistent and positive associations between suboptimal DNA repair capacity and cancer occurrence (64).

NOTE: This figure is included on page 12 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.3 Causes, cellular responses and consequences of DNA double-strand breaks (based on van Gent et al. 2001)

1.2.3 Nutrition and Colorectal Cancer

The relationship between specific dietary components and cancer development and

progression has been of great interest for decades. The complexity of diets and the fact that dietary components may modify one or many steps in the cancer process makes simple recommendations difficult (65, 66).

Epidemiological studies indicated that a diet rich in fruits and vegetables is associated with lower incidence of various forms of cancer (67, 68). Many components of fruits and vegetables may be responsible for their protective effect; such as micronutrients, phytochemicals and fibre. Deficiency in micronutrients including folic acid, Vitamin B12, B6, C, E, iron, niacin and zinc have been shown to mimic radiation by causing DNA damage (69-72). Major phytochemicals found in fruits and vegetables are terpenoids (including carotenoids), phenolics, nitrogen containing alkaloids and sulphur compounds (73). Many carotenoids are antioxidants, which can help to protect the body against damage caused by oxygen free radicals and phenolic compounds are known to provide protection via manipulation of phase I and II enzymes (73).

Although there is no internationally accepted definition of dietary fibre, its composition and actions may be described as:

Dietary fibre means that fraction of the edible part of plants or their extracts or synthetic analogues that – (a) are resistant to digestion and absorption in the small intestine, usually with complete or partial fermentation in the large intestine; and (b) promote one of the following beneficial effects – (i) laxation (ii) reduction in blood cholesterol (iii) modulation of blood glucose and, (c) includes polysaccharides, oligosaccharides (degree of polymerisation >2) and lignins. (74)

This definition is important as it shows its range and complexity. Although there have been mixed views on the protective effects of fibre on CRC risk (51, 75-80), recent studies indicate that there is a strong inverse link between fibre consumption and CRC risk (78,

81, 82). A large European prospective study, involving over 500,000 subjects, has shown an inverse relationship between dietary fibre intake and the incidence of large bowel cancer (81). Moreover, a majority of correlative studies in humans showed either a strong or moderate protective effect of dietary fibre or fibre-rich foods on CRC (83). Another recent epidemiological study from Japan, utilising data from over 40,000 subjects, found a decreasing trend in risk of CRC with increasing intake of total dietary fibre (82).

In contrast, epidemiological studies indicate that a diet high in fat and dietary protein may increase risk (84, 85). Present data have linked a high dietary intake of ω-polyunsaturated fatty acids (PUFA) such as linoleic acid to increase risk of CRC (86). Moreover, human and animal studies have demonstrated a positive association between CRC risk and diets high in red or processed meat (84, 87-91). The evidence from both human and animal experiments regarding the influence of dietary protein on cancer risk, both quantitatively and qualitatively, suggest the quality of protein had no influence on colon cancer risk, while increasing concentrations of protein in the diet did significantly increase risk (92). Another rodent study using carcinogens to induce tumours showed that protein type influenced CRC risk (93).

This thesis focused on two major nutritional components on the large bowel health: dietary protein and fibre.

1.3 Influence of Dietary Protein on Large Bowel Health

In recent years, the increase in obesity has become an enormous concern for the communities around the world. This has lead increasing numbers of people to adopt high protein/low carbohydrate diets for weight control purposes. Although there is strong

evidence that this type of dietary method helps weight loss, a greatly increased consumption of protein may raise concern for large bowel health.

In general, a high fat diet is considered to be harmful for bowel health (94, 95). However, the effects of dietary protein on bowel health are controversial (96). One report suggested that high protein diets (24% of energy intake) could offer significant reductions in risk factors for heart disease as well as enabling weight reduction (97). It is known that increased dietary protein intake enables more undigested protein from the small intestine to reach the large intestine. This had led to the development of the concept of resistant protein by analogy with RS (98). A recent rodent study, however, demonstrated that resistant protein promoted its fermentation which enhanced tumourigenesis (99).

A meta-analysis of prospective population studies identified that protein *per se* was an independent risk factor for CRC (100). Another study suggested that high protein consumption may be harmful for bowel health due to the increased flow of nitrogen to the gut (101). Approximately 2 g of nitrogen enters the large bowel daily mainly in the form of protein, peptides and amino acids, some of which is from endogenous sources. Nitrogen flow from the ileum can be increased by greater protein intake, heat treatment of dietary proteins (to reduce digestibility) and the physical form of foods (101, 102). The main product of bacterial metabolism of nitrogenous residues is ammonia. Ammonia has been suggested to promote tumorigenesis by stimulating cell proliferation (103), which favours the growth of malignant cells (104). In any event, where carbohydrate is rapidly depleted and becomes unavailable, the carbohydrate-to-nitrogen content of the colon decreases and fermentation becomes ‘more and more’ proteolytic. This results in an increase in ammonia production (105). High dietary protein consumption would aggravate the situation by contributing to the production of amines and ammonia (106). In addition, diets high in

meat (such as those recommended for weight loss) generally provide relatively little starch and NSP. This can also lead to increase in ammonia concentrations partly by an increase in available nitrogen and limited fermentation which lowers the bacterial demand for growth (107). Therefore, undigested dietary protein may also contribute to the increase of ammonia in the colon. It has been estimated that with a typical Western diet, up to 12 g of protein per day can escape digestion (108).

There are many different sources of dietary protein and most have different amino acid compositions. It follows that, if the amino acid profile is a factor in CRC risk, protein sources may differ. Furthermore, the methods of preparation (including cooking) of these protein sources must also be considered. One study demonstrated that different cooking methods have a significant influence on large bowel health (109). Generally, it is thought that broiling may be especially deleterious through the formation of heterocyclic amines (ie potential tumourigenic agents). The following section of this thesis explores the differences between various protein sources and possible mechanisms in which these protein sources may influence large bowel health.

1.3.1 Red Meat

Red meat refers to mammalian meat that appears red before cooking (although the actual colour intensity may vary). This includes beef, veal, lamb, pork and buffalo meat. In contrast, animal proteins derived from birds, fish, crustaceans and reptiles are not red meat, although some do appear reddish in colour. It is the presence of myoglobin, which is an intramuscular oxygen carrier, that gives red meat its characteristic colour.

The effect of red meat on large bowel health has been established rather more

clearly than for other dietary protein sources (87). Red meat has been proposed as a factor likely to increase the risk of CRC through several mechanisms (110). High meat consumption is known to increase levels of amino compounds and nitrosamines. It also alters the large bowel flora and changes enzyme patterns (111, 112). These factors may be either cytotoxic or genotoxic themselves or increase the formation of genotoxic compounds in the gut lumen (113). A rat study demonstrated an increase of 33-59% in the incidence of mature rats with dimethylhydrazine-induced intestinal tumours when barbecued beef was substituted for whey protein concentrate against a high fat diet background (96). Significantly increased urinary phenol and *p*-cresol concentrations were also observed with the 'beef fed' rats relative to 'whey protein fed' rats. Phenol and *p*-cresol have been identified as toxic/mutagenic metabolites associated with increased risk of cancer (107, 114-116). Some studies suggest the association of red meat with the risk of colon cancer may be due to its fat content (117, 118). However, other hypotheses suggest that the current Western diet which is relatively high in meat augments rates of colon cancer by increasing the faecal concentration of endogenous nitrosamines (119), carcinogenic tryptophan metabolites (120), or carcinogens resulting from the cooking of meat (121). Cooking of the meat is thought to be critical to large intestinal health as it was shown in a study that pan-broiling of beef increased mutagenic activity compared to microwave irradiation (122).

Although recent epidemiological studies suggest that there are strong correlations between red meat consumption and CRC risk (89-91), the proposed mechanisms by which red meat causes this damage remain unproven. The following section explores four major mechanisms by which red meat may increase CRC risk.

1.3.1.1 Heterocyclic amines

Heterocyclic amines (HCA) are carcinogenic compounds created by the pyrolysis of creatine, amino acids and monosaccharides which can occur during the cooking process of any protein with the appropriate amino acid composition. *In vitro* experimentation has shown that HCA caused DNA mutations (123). Studies in animal models demonstrated that HCA induced tumours at various sites including the colon (124). In addition, a rodent study has shown that APC mutations were induced when PhIP (2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) (the most abundant HCA generated in cooking) was added to the diet (125). Hence, HCA are suggested to be involved in the initiation process of carcinogenesis, as APC gene mutation occurs in 85% of all sporadic and hereditary colorectal tumours in humans (126).

1.3.1.2 N-nitrosocompounds

N-nitrosocompounds (NOC) are among the most toxic chemical carcinogens (127, 128). A small amount in the human body could induce a significant increase in CRC risk. NOC can be produced endogenously in the colon by a two step process. Amino acids are converted into amines and amides by bacteria in the colon, which then undergo N-nitrosation to form NOC (60). The amino acids contained in red meat, but not white meat, are thought to be important in the formation of NOC as red meat concentration in the diet is directly related to the concentration of NOC in the faeces (129, 130). Furthermore, with high red meat consumption, the concentration of NOC are of the same order as the concentration of tobacco-specific NOC in cigarette smoke (129). In addition, a clinical

trial suggested that it is heme iron, not protein residues or inorganic iron, that stimulates endogenous NOC production (131).

1.3.1.3 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are formed by incomplete combustion of coal, oil, petrol, tobacco, meats or other organic materials. Exposure to PAH occurs commonly from consumption of pyrolysed foods or inhaled cigarette smoke (132). Although there are over 100 types of polycyclic aromatic hydrocarbons (PAH), extensive study is conducted only for benzo(a)pyrene (BaP) (133). A previous rodent study has shown that when BaP is given to rodents as part of their diet, tumours developed in the forestomach, oesophagus and tongue (134). However, since quantification of individual PAH is difficult and intake of BaP can occur from tobacco, pollution and other burnt foods, it is inappropriate to assume that dietary meat consumption contributes to the majority of PAH intake. Therefore, the contribution of PAH from the diet to CRC risk remains unclear.

1.3.1.4 Heme Iron

Babbs (1990) (135) suggested that high amounts of unabsorbed faecal iron, resulting from excessive dietary iron, may catalyse oxygen radical production. It is thought that unabsorbed iron increases faecal mutagenicity, activating carcinogens or tumour promoters within the large intestinal lumen. However, there is mixed evidence

regarding the association between high dietary iron concentration and colon cancer risk (136). Sesink *et al.* (1999) (137) investigated the different effects of dietary iron sources on risk markers of colon cancer in a rat model. They showed that heme iron increased cell proliferation, lipid peroxidation and faecal water cytotoxicity in comparison to ferric citrate, protoporphyrin IX and bilirubin. Moreover, carcinogen induced cancer studies in rodents showed that heme iron intake increased aberrant crypt foci (ACF) size and also increased tumour counts (138, 139).

1.3.2 White Meat

White meat refers to any light-coloured meat, such as fish, seafood, and particularly chicken. Examples of white meat proteins include proteins derived from birds, fish, reptiles, crustaceans or bivalves. However, meat that turns white when cooked, like pork, are not considered white meat. Epidemiological studies suggest that the effect of white meat consumption on CRC risk is low (118, 140). However, a 6-year prospective study suggested that both red meat and white meat contribute to increases in CRC risk (141). In a clinical study examining white meat consumption and NOC concentrations, subjects consuming 420-600 g per day white meat had significant reduction in concentration of NOC compared to subjects consuming 420-600 g per day red meat, but no significant difference compared to subjects on a 60 g per day red meat diet (130). Although there are conflicting views on white meat consumption and CRC risk, white meat is still regarded as a low risk food component with regard to CRC.

1.3.3 Casein

Casein is a dairy protein which makes up about 75-80% of all milk protein. It is heat stable and often used to nutritionally fortify foods and as a dietary supplement, due to its high protein content and quality. Casein and caseinates are used as extenders and tenderizers in imitation sausages, soups and stews. They are also included in coffee whiteners, sauces, ice cream, salad dressing, formulated meat, bakery glazes, and whipped toppings. In addition, casein is the recommended protein source in the standard American Institute of Nutrition (AIN) rodent diets. Although no extensive epidemiological studies have been conducted on casein and CRC, animal studies indicate that high consumption of casein may be linked with CRC (142, 143). Several studies have shown that cooked casein promotes colon cancer in rats and mice (142, 144, 145). Furthermore, one of these studies indicated that cooked casein caused mucosal abrasion and suggested that this may be the reason for the colon cancer promotion (142). In addition, a previous rodent study showed that there was an increase in colonic DNA damage when dietary composition of casein was increased (143).

1.3.4 Whey Protein

The main dietary source of whey protein comes from cows' milk, which contains 0.8 g of whey protein per 100 ml, and ricotta cheese which contains 10 g per 100 g. Recently whey protein has been added to increasing numbers of dietary products due to its manufacturing properties such as its gelling strength and antioxidant properties. The

tastelessness of whey protein encourages manufacturers to add it into their product since it does not influence the flavour of the final product. Whey is commonly included as an ingredient in bakery goods, bread, ice cream, frozen desserts, non-fat powder milk and cream fillings.

Whey contains a high proportion of water and can be isolated by ultra-filtration, spray drying and evaporation. Non-protein components of whey can be removed by ion-exchange or micro-filtration, which removes the majority of the fat, lactose and denatured proteins. 70-80 % of the total whey protein is primarily composed of two small globular proteins, ß-lactoglobulin and α-lactalbumin. Other minor components of whey protein include lactoferrin, immunoglobulins, glycomacropeptide, bovine serum album and phosopholipo-proteins. There are limited numbers of published dietary interventions studies investigating whey protein. However, increased use of whey protein has increased attention on its effect on factors such as cancer. To date two studies have examined the effect of whey protein on urogenital, pancreas, liver, and breast cancers (146, 147). Bounous *et al.* (2000) (147) indicated that dietary supplementation of whey has anti-tumour effects in urogenital cancer. However, a phase I-II clinic study on pancreas, liver and breast cancer did not result in any clear conclusion (146). Both studies suggest possible anti-carcinogenic effects of whey protein.

Findings from animal studies have led to a number of speculations on how whey protein may inhibit the carcinogenesis process. The most likely mechanism is the stimulation of cellular glutathione concentrations. The tri-peptide glutathione (GHS) plays an important role in enzyme activity, and metabolic and cell cycle related functions in all cells (148). GHS and glutathione transferase are major components involved in the metabolism of xenobiotics (149). A primary process to increase the GHS is through

increased delivery of cysteine to a cell. Whey protein is the richest protein source for this amino acid and effective supply of cysteine may increase GHS concentrations in cells (150).

1.3.5 Soy Protein

As one might expect, soy protein comes from soybeans, a legume from the pea family. Soy plants store protein in their seeds for development of the embryo. These and other legume seed storage proteins belong to the globulin family of such proteins called leguminins and vicilins. Soybeans also contain other proteins such as prolamin, trypsin inhibitors, hemagglutinins and cystein protease. Soybeans are processed into three types of protein products; soy flour, soy concentrate and soy isolate. Soy protein concentrate is soybean without water soluble carbohydrates and contains approximately 70% soy protein. Soy protein concentrate retains most of the fibre of the original soybean. Soy isolate is the most refined form of soy protein and consists of approximately 90% protein and 10% other components. It is derived from defatted soy meal, which has the majority of fats and carbohydrates removed. Soy flour is made by grinding soybeans into a fine powder. Soy protein in various forms is used in a variety of foods such as imitation meats, soups, salad dressings, cheeses, frozen desserts, pasta products and cereals.

The majority of epidemiological studies examining soy consumption and cancer have focused on breast and prostate cancer, both of which are hormone sensitive. However, previous studies have suggested that CRC may also be hormone sensitive (151, 152). A clinical trial showed that CRC risk is reduced in women by the use of post menopausal hormone therapy (152). Soybeans and most soy-based products contain

phytochemicals; in particular, isoflavones, which are structurally similar to endogenous estrogen and bind to estrogen receptors (153). These isoflavones and other phytochemicals have been shown to inhibit cancer cell growth and induce apoptosis (154). However, several rodent studies demonstrated that consumption of soy protein induced damage to the colonic epithelium and stimulated proliferation of colonocytes when compared with a casein diet (155, 156).

Furthermore, results from a meta-analysis of population studies indicated that the relationship of soy consumption and cancer risk may depend on whether or not the soy food was fermented or not (157). For example, the risk of stomach cancer was significantly lowered when high amounts of unfermented soy foods (soy milk, tofu and soybeans) were consumed. However, the risk increased with diets containing high amounts of fermented soy foods (miso, fermented soybeans, and soy paste). Similar results were obtained in a more recent meta-analysis by Spector *et al.* (2003) who suggested that an inverse relationship between unfermented soy consumption and rectal cancer onset but not fermented soy products (158).

There are contradictory findings on the risk of CRC and consumption of dietary soy (154-156, 159). Moreover, lowering of plasma cholesterol by soy in comparison with casein indicates that there are different nutritional properties of soy protein compared to other protein types. Therefore, further investigation is needed to elucidate the effects on soy on the large bowel health.

1.4 Dietary Fibre

Fibre is a heterogeneous group of (largely) plant-derived material. As indicated

previously, these are principally carbohydrates and share the characteristic of being indigestible by human small intestinal enzymes. Also as noted, there is no internationally accepted definition of fibre, dietary fibre is generally considered as complex carbohydrates that reach the colon (160, 161). These complex carbohydrates include a range of non-starch polysaccharides (cellulose, hemicellulose, pectin, gums, and mucilages) and starches which resist digestion (74). Dietary fibre is subclassified into two types, soluble and insoluble. The soluble fibres, such as those found in fruits and oats, tend to be highly fermented in the proximal colon including the caecum (162, 163). The insoluble fibres, such as wheat bran, are more slowly fermented and have a greater impact on fermentation events in the distal colon and are detected more readily in faeces (162, 163).

It would be expected that dietary fibre consumption is likely to lead to a reduction of the risk of CRC. However, the extent and mechanism of protection by fibre has not been fully characterised. Several theories have been proposed as shown in **Table 1.1**. Originally, the protective mechanism of dietary fibre was thought to be due to dilution and binding of toxins and carcinogens in the intestine through its physical presence (164). Increased faecal bulking from fermentation reduces intestinal transit time. This leads to the reduction in mucosal exposure to potential carcinogens or tumour promoters (165, 166). The basic fermentative reaction in the human colon consists of hydrolysis of polysaccharides, oligosaccharides, and disaccharides to their constituent sugars which are then fermented resulting in an increased biomass (167). Fermentation yields metabolisable energy for microbial growth and maintenance (168). Instability of the colonic fermentation from a diet high in fat and protein but low in fibre is considered to cause an irregularity in the mass of digesta in the colon, which induces fluctuations in colonic fermentation and may increase the possibility of the production of cytotxic agents (and loss

on protective ones) leading to greater risk of colorectal carcinogenesis (106).

More recently, there has been increased focus on the prebiotic action of fibre on large bowel health. Until now, the prebiotic functions of fibre and their correlation to CRC have not been adequately explored. Fibre exerts marked changes to the luminal environment and colonic microflora (74) and it has been shown that dietary fibre increases the total mass of bacteria but leads to decreases in secondary bile acid production (169).

Large bowel carbohydrate fermentation lowers digesta pH through direct acidification and the fixing of nitrogen in bacterial mass (74). It is proposed that this reduces the potential tumour promoter activity of secondary bile acids. The enzymatic activity of 7α-dehydroxylase, which coverts primary bile acids to secondary bile acids changes with reduced pH (170). In addition, a human population study has shown that subjects with the lowest faecal pH have the lowest rates of colon cancer (171). Fibre significantly increases concentration of SCFA and this partially contributes to the lowering of pH. The effect of SCFA, butyrate in particular, is discussed in more detail later in this thesis.

Another possible protective mechanism of fibre is the prevention of CRC via modulation of lifestyle factors (172). It was suggested that the lifestyle factors that cause insulin resistance and hyperinsulinaemia, stimulating the growth of CRC cells. There is some *in vitro* evidence that insulin and insulin-like growth factors (IGF) may influence CRC risk and the risk for developing CRC increases by 40% in diabetes (173). This therefore suggests that further investigation is required.

Population studies have suggested that intake of fibre is linked to reduction of CRC risk (174-176). This led to the initial assumption that non-starch polysaccharides (NSP), major components of dietary fibre, have a significant protective contribution against CRC.

However, a recent study showed that NSP may not be ae important a contributor to protection as RS (100).

Table 1.1: Possible Mechanisms for the protective action of dietary fibre on colorectal oncogenesis (based on Young et al. 2005)

NOTE: This table is included on page 27 of the print copy of the thesis held in the University of Adelaide Library.

1.5 Resistant Starch

Starch is a substantial component of most human diets. Historically, starch has been thought to be fully digested to glucose in the small intestine as very little starch is normally found in faeces (74). However, this is now known to be incorrect. RS is an undigested starch which reaches the large bowel and is fermented by colonic microflora, thereby contributing to the optimal function of the large bowel through the production of SCFA (168, 177). RS functions similarly to fibre and many nutritionists are of the opinion that it

should be classified as a component of dietary fibre. RS is found in intact (unprocessed) wholegrain cereals and some seeds and nuts, for example oats, rye, wheat, barley, semolina, corn, linseed and sesame seeds. Processed starchy foods such as some breakfast cereals, white bread, rice and pasta also can contain RS. Recently, some processed foods including breads and cereals have been supplemented with RS in the form of a specific high amylose starch (Hi-Maize™), which is derived from corn. Furthermore, foods such as legumes, for example lentilis, baked beans and unripe fruit (especially bananas), contain RS. In addition, cooked cold rice (such as sushi rice) cold pasta salad and cold boiled potato salad are high in RS.

RS is classified into four groups (74, 178) (**Table 1.2**). RS_1 includes starch that is trapped within whole plant cells and food matrices where there is a physical barrier to amylolysis (conversion of starch to sugar by enzyme and acids). In raw starch granules, starch is tightly packed in a radial pattern and is relatively dehydrated. RS_2 comprises of those granules from certain plants that are gelatinised poorly and hydrolysed slowly by α-amylases. RS_3 represents retrograded starch, where the starch granule is partially or completely hydrated through cooking in water and is allowed to stand. This leads to reassociation of the polymer chains leading to RS formation. Examples of RS_3 starches include cooked and cooled rice or potato. RS_4 comprises the chemically modified starches obtained by chemical treatments, like di-starch phosphate ester, to improve the functional characteristics of starch.

Table 1.2. Classification of types of resistant starch (RS), food sources, and factors affecting their resistance to digestion in the colon

Type of RS	Description	Food sources	Resistance Minimized by
RS_1	Physically protected	Whole or partly milled grains, seeds and legumes	Milling, chewing

RS_2	Un-gelatinised resistant granules with type B crystallinity, slowly hydrolysed by α-amylase	Raw potatoes, green bananas, some legumes, high amylose corn	Food processing and cooking
RS_3	Retrograded starch	Cooked and cooled potatoes, bread, cornflakes, food products with repeated moist heat treatment	Processing conditions
RS_4	Chemically modified starches due to cross-linking with chemical reagents	Foods in which modified starch have been used (for example, breads, cakes)	Less susceptible to digestibility *in vitro*

Starch is made up of glucose molecules linked together to form amylose and amylopectins. Amylose has a linear structure and can form tightly packed granules, which are insoluble and difficult to digest. However, amylopectin has a branched structure and thus can not form tightly packed granules and is, therefore, easier to digest. Most plants contains about 20-25% amylose. However, some plants, such as peas, have 60% amylose and certain species of maize, such as Hi-Maize™, can have up to 80% amylose.

Previously it had been assumed that non-starch polysaccharides (NSP), a major fibre component, were the principle fermentative substrates. A population study showed that individuals generally consume less than 20 g of NSP per day (179). These values are well below the 60-80 g substrate per day required to sustain the 10^{13}-10^{14} organisms found in the normal human large bowel. It was speculated that this gap was most likely filled by RS (179). A clinical study conducted in South Africa demonstrated that RS consumption may provide more protection against CRC risk than NSP (180). The incidence of CRC and other large bowel diseases in the South African black population is much lower than in the white population (181). The dietary fibre consumption of the urban black population tested was lower than in the white population, but starch intake of the urban black population was significantly higher (182). It was also demonstrated that a typical diet eaten by the black population showed increased SCFA and reduced stool pH compared to a

typical diet consumed by white populations (180). Therefore, these studies suggest that the black population diet is better than the white with regard to CRC risk. This was supported by an epidemiological study which showed that RS is more protective against CRC risk than NSP (100).

The experimental evidence supports protective effects of RS against large bowel diseases. A randomized crossover study has shown that daily consumption of RS (approximately 40 per day) significantly increased the daily excretion of faecal nitrogen and decreased faecal concentrations of both ammonia and phenols (166). Ammonia is thought to promote tumourigenesis by stimulating cell proliferation and favouring the growth of malignant cells, while phenols (*p*-cresol and phenol) by-products from the metabolism of aromatic amino acids, are known for the promotion of skin, bladder and bowel cancers (103, 114-116). Epidemiological studies support the protective role for starch (as RS) in CRC (84, 168). However, the animal experiment data with RS in chemically-induced carcinogenesis are somewhat inconclusive (183).

1.5.1 Gut Microbiology

The intestinal ecosystem is characterised by dynamic and reciprocal interactions between host and its microflora. However, despite the fact that the importance of gut microflora for human health is being increasingly recognised, the underlying mechanisms of these interactions are very complex. Nonetheless, evidence obtained through animals raised in germ-free conditions have provided important information about the physiological effect of the microbial community in the gut (184). Main functions of gut flora include fermentation of non-digestible dietary residues and endogenous mucus,

salvaging of energy as SCFA, control of epithelial cell proliferation and differentiation, development and homeostasis of the immune system and protection against pathogens (185).

The interaction between dietary factors occurs in the lumen of the large bowel (186). It is possible that dietary carcinogenic effects could be mediated by changes in the metabolic activity and composition of gut microflora. Intestinal bacteria potentially play a part in the initiation of colon cancer through the production of carcinogens, co-carcinogens, or procarcinogens (187). Bacteria, such as bacteroides and the *clostridium* genera increase the incidence and growth rate of colonic tumours induced in rodents (188, 189). In contrast, *lactobacillus* and *bifidobacteria* prevent tumourigenesis (190, 191). Although the evidence is inconclusive, colonic microflora seem to be a critical environmental factor in the modulation of CRC risk.

RS is known to change the composition of the intestinal microflora by stimulating the growth of various bacteria including *bifidobacteria*, *lactobacilli*, *eubacteria* and *streptococci* (192). Lower large intestinal pH results from higher SCFA formation and this affects bacterial populations. The enhanced counts of *lactobacilli* inhibit the growth of pathogenic bacteria such as certain *E-coli* strains, *clostridium* difficile, or sulphur/sulphate reducing anaerobic bacteria (193).

1.5.2 Fermentation

Fermentation is an anaerobic redox process, in which the oxidation of the substrate is coupled to the reduction of another substrate or an intermediate derived from the oxidation, with the difference in redox potential of the substrate and the end product

providing energy for ATP synthesis (194). The rate, site and extent of dietary fibre/RS fermentation in the gut is dependent on a number of factors including solubility, chemical structure, availability of other more readily fermentable substrates and the composition of the colonic microflora. Insoluble fibre is resistant to colonic microflora fermentation and contributes to faecal bulk.

Fermentation has a number of important consequences in large bowel physiology and possible protection against CRC. Increases in bacterial cell mass, caused by fermentation, results in an increase in faecal weight. With the increase in faecal weight, transit time is reduced and the large bowel contents are diluted. This reduces the time putative carcinogens are in contact with large bowel mucosa. This effect was originally suggested by Burkitt in 1969 to explain the protective effect of dietary fibre against CRC and has stood the test of time (195).

Increased fermentation is also known to generate an acidic environment with a reduction in pH via SCFA production. Several population studies have shown that subjects with the lowest faecal pH have the lowest grades of colon cancer (171). There are several proposals to suggest how a reduction in colonic pH provides an anti-carcinogenic environment. The acidic environment is known to reduce the potential tumour promoter activity of secondary bile acids. Furthermore, low pH inhibits the activity of the bacterial enzyme, 7α-dehydroxylase, which produces secondary bile acids from primary bile acids (170). Therefore, dietary fibre through acidification of colonic contents is considered protective against colon cancer.

1.5.3 SCFA

Over the past few decades considerable investigation has been undertaken in order to understand the physiological role of SCFA. Previously, they were considered as a major factor in the aetiology of carbohydrate induced diarrhoea. However, the rapid absorption of SCFA and the metabolism by the intestine led to a more general speculation that SCFA are beneficial (196).

Dietary and endogenous residues that reach the colon may be metabolised by anaerobic bacteria to produce SCFA and other substrates such as lactate, succinate, ethanol, hydrogen, methane and carbon dioxide. The predominant substrates that contribute to the SCFA production are polysaccharides, oligosaccharides, protein, sugars and mucus (51). The principal SCFA that result from carbohydrate fermentation are acetate, propionate and butyrate. Other SCFA produced in smaller quantities from colonic fermentation are isobutyrate, valerate and isovalerate.

SCFA contribute to large bowel function and prevent pathology through their luminal actions and metabolism by colonocytes (74). Daily production of SCFA in man is approximately 100-200 mM. The majority of this is absorbed by the colon via diffusion or anion exchange (196). Furthermore, absorbed SCFA are transported via the portal vein to the liver, and the fraction not absorbed is distributed to other body organs and tissues. Although SCFA provide valuable information on large bowel health, quantification of the colonic SCFA remains difficult. SCFA in colonic contents have been determined in colostomy patients and post-mortem samples, but these approaches are impractical for large-scale dietary studies. Faecal measurements are useful in determining the changes in excretion, but not necessarily in production because faecal SCFA can be influenced by rate of transit time (74). Therefore, the majority of experimental data have been obtained from animal models, mainly rats and pigs.

In general, clinical studies have shown that RS increases faecal SCFA concentrations and excretion (197-199). Furthermore, animal studies have shown that feeding of RS raises large bowel total SCFA (200-203), highlighting the strong association between RS consumption and SCFA production in the large bowel. In addition, it was found that consumption of diets high in RS changed large bowel SCFA profiles, and in particular increases in butyrate and propionate (74).

1.5.3.1 Butyrate

Butyrate has been considered as the most important SCFA. It is thought to play a role in maintaining a normal colonocyte population. Fermentation of RS is known to yield a relatively high proportion of butyrate. Butyrate is considered to be an important by-product of RS-metabolising bacteria within the colon (74).

Butyrate has been suggested to decrease the growth of most human colon cancer cell lines by inhibiting cell proliferation and enhancing differentiation and apoptosis (204, 205). β-oxidation of SCFA occurs in the mitochondria to regulate cell proliferation and apoptosis in the intestinal mucosa (206). This was shown using a knock-out mouse model of short chain acyl dehydrogenase, the enzyme responsible for encoding the mitochondrial β-oxidation of SCFA (207). When the ability to efficiently metabolise SCFA was reduced, the level of apoptosis in the colon was significantly reduced.

The potential anti-cancer properties of butyrate in the colon are linked with a small molecule signalling pathway involving butyrate and its cellular target histone deacetylase (HDAC) (208). Butyrate inhibits the enzymatic activity of HDAC, resulting in changes in the transcription of specific genes, including the induction of the cyclin-dependent kinase

inhibitor p21/Cip1/WAF1 (209). Both inhibitions of HDAC and expression of p21 result in cell cycle arrest and differentiation.

However, not all experiments support a chemopreventitive effect of butyrate (210). For example, a previous rodent study showed that administration of butyrate in the drinking water increased the number of colon tumours detected (211). Furthermore, an azoxymethane-induced colon cancer rodent study demonstrated that administration of slow release butyrate pellets did increase the colonic butyrate concentration but found no protective effect (212). Since there is lack of agreement, particularly between *in vivo* and *in vitro* studies, regarding butyrate and colon cancer, this discrepancy is termed the "butyrate paradox".

The effect of butyrate on colon carcinogenesis may be depended upon the timing of butyrate administration in relation to the stage of the cancer development. For example, butyrate inhibits HDAC. This suggests that in its presence, DNA would be in a more open form, which may be optimal if DNA damage has occurred as more repair mechanisms may be initiated to prevent mutations (213). In addition, concentration is another factor which may affect the physiological effects of the butyrate. Several studies have indicated that low concentrations of butyrate stimulate cell proliferation, but high concentration may inhibit it (214).

1.6 Colonic Mucus Barrier

Another defence mechanism of the colon from bacterial and genetic damage is the mucus layer. A mucus layer covers the surface of the gastrointestinal tract, protecting intestinal tissues from pathogens (**Fig. 1.4**). The mucus layer consists of mucins, gel

forming glycoproteins, and a large number of smaller glycoproteins, proteins, glycolipids, and lipids (215). In the large bowel, the layer is especially thick to prevent the large number of bacteria present from infecting tissues (216). Important physiological roles of the mucus include lubrication (217), and a stabiliser for the mucosal association of some enteric bacteria (218). Degradation of the gel layer is likely to result in impairment of these functions. *In vivo* studies have shown that the mucus layer is a dual-layer and protects the mucosa through two mechanisms (216, 219, 220). The outer layer is a non-adherent mucus layer and is believed to reduce the shear stress to the mucosa (221). The constant turnover of this layer may act to trap bacteria, secondary bile acids and endogenous protease. The inner layer acts as a size exclusion barrier to toxic luminal agents. This barrier allows uptake of water, salt and SCFA, while preventing access of larger molecules to the mucosa (220). This dual-layer system is necessary to prevent digestion of the colonic wall by both hosts and bacterial enzymes.

The thickness of the colonic mucus layer is known to be influenced by diet (222). A recent study demonstrated that there was a significant decrease in mucus thickness and secretory response when rats were fed fibre-deficient diets (222). In addition, another rodent study showed that RS contributed to the maintenance of the colonic mucus layer thickness (143). This study further showed that colonic genetic damage was strongly linked with decreased mucus layer thickness.

NOTE: This figure is included on page 37 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.4: The mucosal barrier
The mucosal barrier separates the internal milieu from the luminal environment. Function of the barrier depends on factors such as the endothelium and epithelium lining, mucosal blood flow, reactivity of dynamic defence system, epithelial secretions and immunocompetent cells. (Based on Callicott & Womack 2006)

1.6.1 Mucins and MUC genes

Mucins, which form the major component of mucus, are large carbohydrate-rich glycoproteins and have a high content of cluster oligosaccharides O-glycosidically linked to tandem repeat peptides (mainly threonine, serine and proline) (223, 224). There are two structurally and functionally different classes of mucins: secreted gel-forming mucins

(encoded by the following genes, MUC 2, MUC5AC, MUC5B, and MUC 6) and trans-membrane mucins (MUC 1, MUC3A, MUC3B, MUC4, MUC12, MUC17). Besides their protective function in the normal colon, alternations in mucins are a common feature of colonic neoplasia. Moreover, expression of several MUC genes is linked with colon cancer. MUC 1 expression is increased in colon cancer (223), while several studies have found that expression of MUC2 is generally decreased in colorectal adenocarcinoma (225, 226). Also, MUC2 knock-out mice develop adenomas in the small intestine that progress to invasive adenocarcinoma, as well as rectal tumours (227), suggesting that MUC2 gene is involved in the suppression of CRC. Furthermore, MUC5AC which is normally expressed in the surface epithelium of the normal stomach, and not in the normal colon, is often expressed in adenomas and colon cancers (228, 229).

1.7 Biomarkers of Large Bowel Health

There are two major categories of biomarkers for CRC research. They involve changes in colonic epithelium or changes in faecal composition. Biomarkers of colonic epithelium provide greater information than faecal biomarkers. However, to obtain a sample of epithelium in clinical studies requires invasive methods such as pinch biopsy, or can only be collected at autopsy. Wide-scale screening using faecal occult blood testing results in up to 33% reduction in colorectal adenocarcinoma mortalities, but requires many unnecessary colonoscopies (230-232). Therefore, stool DNA is emerging as a potentially important new approach for the early detection of colorectal neoplasia (233). The most common genetically based faecal tests applies the concepts of chromosomal instabilities with mutations progressively accumulating in the APC, p53 tumour suppressor genes, and

K-ras oncogene (234). Moreover, a recent study demonstrated that exfoliated cells from the surface of the stool can be used to measure DNA adducts (57). However, measurements of human colonocytes via biopsy or autopsy still provide the best indication of large bowel health as exfoliated cells in general have very low cell viability. Other colon cancer biomarkers used in previous studies include proliferation, apoptosis, faecal mutagenicity and total and secondary faecal bile acid excretion.

1.7.1 Proliferation and Apoptosis

A measurement of proliferation provides an indication of how a dietary component or chemical may increase colon cancer risk. Proliferation, a process in which cells divide, increases the chance of genetic errors related to the carcinogenic process. Therefore, proliferation is often linked with risk of cancer (235, 236). In contrast, apoptosis provides information on the way in which cells with genetic mutation can be removed. However, the rate of apoptosis is very low in the colon and meaningful measurements can only be obtained if a chemical carcinogen is administered to increase the rate of apoptosis (237).

1.7.2 Comet Assay

Colonic genetic damage is thought to be prerequisite for sporadic cancer initiation. The alkaline comet assay (single-cell electrophoresis) is used frequently in genotoxicity testing and population biomonitoring (238, 239). This is due to its sensitivity for detecting single-strand DNA breaks, ease of use and rapid performance. Furthermore, this assay

requires a relatively small number of cells compared to other genotoxicity assays. In previous studies, the comet assay has been successfully used to determine DNA damage in rat colonocytes (143, 240). The comet assay is a useful method to measure and quantify DNA damage (strand breaks) in individual cells (241-243) due to the higher mobility of smaller DNA fragments during electrophoresis. The intensity of the DNA tail increases in proportion to the extent of DNA damage (**Fig. 1.5**) (244). A revised version of the method has also been developed to measure double-strand DNA breaks independent of the presence of single-strand breaks (245, 246). Furthermore, specific base damage can be identified by incubating lysed cells with base damage-specific endonucleases before electrophoresis (247). It is these reasons that led to this assay being used in the experiments presented in this thesis.

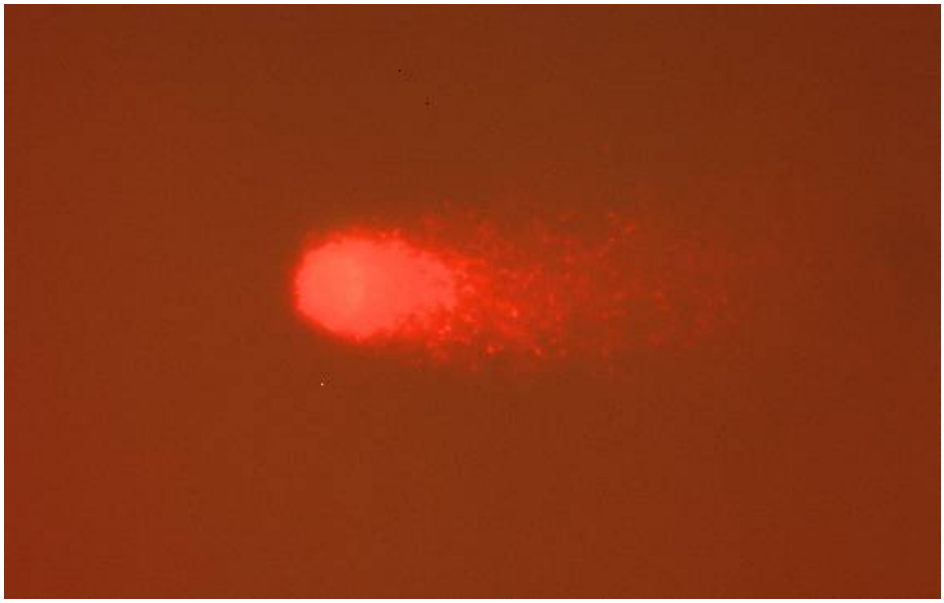

Figure 1.5 Image of a typical cell used for comet assay. 200x magnification, section stained with propidium iodide.

1.8 HYPOTHESES AND AIMS

Recently, there is an increased public interest on high protein diets as means of weight control. However, effects of these diets on large bowel health need better understanding. Epidemiological and experimental evidence suggest that high protein diets have adverse effects on the large bowel. In contrast, dietary fibre, RS in particular, appears to be protective against large bowel diseases. The present thesis investigated the effect of several different types of protein sources (red meat, white meat, soy, whey and casein) on colonic DNA damage and interaction with RS.

1.8.1 General Hypotheses

- Diets high in dietary protein increase colonic DNA damage and reduce the colonic mucus barrier
- Dietary red meat increases colonic DNA damage compared to other protein types
- RS protects large bowel health by increasing colonic fermentation and SCFA production (butyrate in particular)
- RS dose-dependently protects colonic DNA damage
- Red and white meat dose-dependently increases colonic DNA damage

1.8.2 Specific Aims and Objective of This Thesis

The aim of this thesis was to gain insights into how different types of proteins influence large bowel health, with particular respect to CRC risk. To investigate this aim,

animal studies were designed to determine:

1. The effect of red meat on CRC risk and thickness of the mucus layer
2. Whether RS protective against red meat-induced colonic DNA damage
3. Whether special types of proteins (whey and soy) cause similar colonic DNA damage as casein
4. Whether RS protects protein induced colonic DNA damage in a dose-dependent manner
5. Whether white meat increases colonic DNA damage similar to casein and red meat
6. Whether red meat increases colonic DNA damage in a dose-dependent manner
7. Whether butyrate is the major component responsible for the protective effects of RS

CHAPTER 2

RESISTANT STARCH PREVENTS COLONIC DNA DAMAGE INDUCED BY HIGH DIETARY COOKED RED MEAT OR CASEIN IN RATS

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Cancer Biology and Therapy, 2006, 5(3), 267-72

Publication 1:

The aim of this publication was to determine if dietary cooked red meat can cause colonic DNA damage and whether RS is effective in reducing such damage. The rats used in this study were fed diets containing 15% or 25% casein and 25% red meat, with or without 48% high amylose maize starch.

The results showed that red meat diet induced significant higher colonic DNA damage compared to casein diet in the absence of RS. These damages were reversed by inclusion of dietary RS in the diet. Several colonic parameters were measured to identify the changes occurred with these diets. The rats fed RS showed increased digesta weights and SCFA pools (including butyrate) in caecum and faeces, along with lowered pH of the faeces and caecal digesta, and increased weight of the caecum and length of the colon. Furthermore, high dietary casein and red meat reduced the thickness of the colonic mucus layer compared to the low casein diet in the absence of RS. The addition of RS attenuated the loss of colonic mucus thickness induced by both high protein diets.

These results suggest that the inclusion of RS can effectively attenuate the increase DNA damage and reversed thinning of the colonic mucus barrier induced by high protein diets (both red meat and casein). Therefore, food contains RS may provide protection against bowel diseases such as colorectal cancer.

Toden, S., Bird, A.R., Topping, D.L. and Conlon, M.A. (2006): Resistant Starch Prevents Colonic DNA Damage Induced by High Dietary Cooked Red Meat or Casein in Rats.
Cancer Biology and Therapy, v. 5 (3), pp. 267-272, March 2006.

CHAPTER 3

DIFFERENTIAL EFFECTS OF DIETARY WHEY, CASEIN AND SOY ON COLONIC DNA DAMAGE AND LARGE BOWEL SCFA IN RATS FED DIETS LOW AND HIGH IN RESISTANT STARCH

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British Journal of Nutrition, 2007

Publication 2:

The results shown in the Publication 1 provided evidence that consumption of a high protein diet, as both casein and red meat, increases colonic DNA damage. It also highlighted that this damage was attenuated by the addition of RS in the diet. This increase in colonic DNA damage induced by high protein diets supports the findings of a meta-analysis study that a high protein diet increases risk of colorectal cancer (100). However, the role of dietary proteins in cancer aetiology in general is poorly understood and it remains to be established whether other protein types have similar effects. Therefore, the aims of this second publication was to determine whether high dietary dairy (casein or whey) or plant (soy) protein have adverse effects on the large bowel and whether dietary RS was protective against these different protein sources.

In this experiment the effects of three different types of dietary proteins, casein, whey and soy were examined with relation to colonic DNA damage, colonic mucus layer thickness and SCFA levels in the caecum and faeces of rats. High dietary casein and soy (but not whey) increased colonic DNA damage. DNA damage was highest with soy when fed 15 or 25% protein in the absence of RS. Caecal total SCFA and butyrate pools were higher in rats fed RS compared with digestible starch.

These results suggest that diets high in protein affected colonic DNA damage and the mucus layer thickness. However, proteins differ in their effects on these indices. Furthermore, these protein induced changes were reversed by inclusion of dietary RS. Therefore, RS has protective effect against the colonic DNA damage produced by variety of protein sources.

Toden, S., Bird, A.R., Topping, D.L. and Conlon, M.A. (2007) Differential effects of dietary whey casein and soya on colonic DNA damage and large bowel SCFA in rats fed diets low and high in resistant starch.
British Journal of Nutrition, v.97 (3) pp. 535-543, March 2007

CHAPTER 4

DOSE-DEPENDENT REDUCTION OF DIETARY PROTEIN-INDUCED COLONOCYTE DNA DAMAGE BY RESISTANT STARCH IN RATS CORRELATES MORE HIGHLY WITH CAECAL BUTYRATE THAN WITH OTHER SHORT CHAIN FATTY ACIDS

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Cancer Biology and Therapy, 2007

Publication 3:

The first two publications have demonstrated the effects of various protein types on colonic DNA damage and the reversal effects of RS. In these studies, 48% HAMS diets were used as the source of RS, but 48% HAMS is too high for human to consume as diets. This next publication sought to determine whether there was a dose relationship between RS intake and amelioration of the effects of high dietary protein in the form of casein. Rats were fed a diet containing 25% casein with 0%, 10%, 20%, 30% or 40% HAMS for 4 weeks, as shown to produce significant increase in colonic DNA damage in publications 1 and 2. The single-strand comet assay was used to determine the colonic DNA damage and this was combined with the use of endonuclease III to ascertain whether formation of 8-hydroguanosine was involved.

Colonic DNA damage was greatest and the mucus barrier was thinnest in rats fed 0% HAMS. DNA damage was reduced and thickness of the mucus barrier increased in a logarithmic dose-dependent manner by HAMS. However, there was no significant difference in 8-hydroguanosine between dietary groups indicating that 8-hydroguanosine formation was independent of dietary HAMS level. As expected caecal and faecal SCFA pools were elevated with increased levels of dietary HAMS.

This publication extends current literature to confirm the protective effects of RS through SCFA production, most likely through butyrate production. Furthermore, inclusion of 10% HAMS was found to be sufficient to oppose colonic DNA damage, and to increase caecal and faecal SCFA.

Toden, S., Bird, A.R., Topping, D.L. and Conlon, M.A. (2007): Dose-Dependent Reduction of Dietary Protein-Induced Colonocyte DNA Damage by Resistant Starch in Rats Correlates More Highly with Caecal Butyrate than with Other Short Chain Fatty Acids.
Cancer Biology and Therapy, v. 6 (2), pp. 253-258, February 2007.

CHAPTER 5

HIGH RED MEAT DIETS INDUCE GREATER NUMBERS OF COLONIC DNA DOUBLE-STRAND BREAKS THAN WHITE MEAT IN RATS: ATTENUATED BY HIGH AMYLOSE MAIZE STARCH

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Carcinogenesis, 2007

Publication 4:

The previous published chapters have shown that increases in dietary protein, as red meat, casein or soy, increased colonic DNA damage in rats. These damages were prevented when RS was added to the diet. The aims of this final publication were to determine the genotoxic burdens of dietary red and white meats on large intestine by measuring single-strand DNA breaks (SSB) and double-strand DNA breaks (DSB) and whether addition of RS in the diet provides protection.

Until now there have been limited studies on the effects of the high dietary red meat on colonic DNA damage. Recent epidemiological studies indicated that high red meat consumption increases the risk of colorectal cancer, but not white meat (87, 89, 91, 117). In contrast, dietary fibre is thought to have protective effects (81). The previous publications presented have shown that high levels of dietary protein significantly increased colonic DNA damage as SSB. In this publication we measured SSB and DSB, a more potent genetic damage. DSB differs from other types of DNA damage as both DNA strands of the double helix are damaged, which can be a potent inducer of chromosomal aberrations. Rats were fed diets containing 15, 25 or 35% of cooked beef or chicken at levels to provide equivalent amounts of protein as beef (13, 22 or 30%), and with or without 20% HAMS for 4 weeks.

Increased consumption of protein as either red or white meat increased colonic DNA damage. However, there was a significantly greater colonic SSB and DSB damage for the rats that consumed red meat. This increased colonic DNA damage was attenuated by the addition of RS in the diet. Thinning of the mucus barrier was again observed with high protein diets and this was reversed with addition of dietary RS. Caecal and faecal measurements indicated that RS consumption increased SCFA pools in caecam and faeces.

This publication demonstrated that dietary red meat had significantly greater

genetic damage than white meat, which corresponds with finding of epidemiological studies. Furthermore, the results of this publication again confirm that RS can effectively reduce the colonic DNA damage caused by high protein diets.

Toden, S., Bird, A.R., Topping, D.L. and Conlon, M.A. (2007) High Red Meat Diets Induce Greater Numbers of Colonic DNA Double-Strand Breaks than White Meat in Rats: Attenuation by High Amylose Maize Starch.
Carcinogenesis, (published online on October 4, 2007)

CHAPTER 6: CONCLUSIONS

The aim of these studies was to compare the deleterious effects of high levels of specific dietary proteins on the integrity of the colonic mucosa of a model animal species, the rat. The studies also aimed at determining the protective effect of resistant starch (RS) fed as a high amylose maize starch against protein-induced damage. As the chapters in this thesis are presented in a manuscript format, the conclusions and implications of the 4 major publications presented in this thesis are discussed below.

6.1 Conclusions and Implications

6.1.1 Differential Effects of Various Protein Types

The proposition that greater protein intake was linked to increased CRC risk has been strengthened by several recent, large epidemiological studies showing that consumption of red meat correlated with higher rates of CRC (89-91). The original hypothesis of this thesis was that the increased risk of CRC is associated with red and processed meat may be a result of increased consumption of protein *per se*. The study described in Chapter 2 of this thesis demonstrated for the first time that consumption of high levels of dietary protein, as red meat, increased colonic DNA damage compared to equivalent levels of casein. In humans these effects could translate to increased risk of CRC. Furthermore, there was a thinning of the mucus layer correlated with increased colonic SSB DNA damage. This is a particularly important finding as it could link to IBD where breakdown of the mucus barrier occurs leading to greater potential for bacterial translocation and an inflammatory response. IBD is also a risk factor for CRC.

Collectively, these data show that dietary protein could increase genetic damage in the absence of a cytotoxic agent such as AOM. However, the data show also that the effect was due to protein and not to red meat alone.

In order to determine whether various protein sources have differential effects on the integrity of colonocyte DNA, the experiments reported in Chapter 3 of this thesis extended the study described in Chapter 2. Whey and soy protein were substituted for red meat as protein sources specifically to compare a plant protein isolate with another protein of animal origin. The absence of any loss of colonocyte integrity with whey protein confirmed previous rodent studies indicating that it could provide protection for the large bowel (93, 248). Soy protein is often regarded as a health food product (249), and is used by vegetarians as a dietary protein source. In contrast to whey, greater dietary soy protein increased genetic damage. Therefore, these current results indicate that whey protein is more protective against colonic DNA damage compared to other protein sources, such as casein, soy or red meat. This is supported by a previous study using rodents with chemically-induced cancer which showed whey protein to be the most protective against tumour formation and soy protein to be the least protective compared with casein and red meat (250). Furthermore,a rodent study of AOM-induced tumours demonstrated that bovine lactoferrin, a major whey component, reduced colon carcinogenesis with a significant lowering in adenocarcinoma incidence (251). One of the possible protective mechanisms of whey is its capacity to increase tissue glutathione. Whey protein can raise colonic glutathione concentration in rats and this may facilitate deactivation of xenobiotics via glutathione transferase activities (147, 156).

While the consumption of whey protein may be beneficial for large bowel health it is highly unlikely that people will consume enough to produce these effects independently.

However, whey protein is usually consumed in supplements, especially for weight control and body building. Under these circumstances enough might be consumed where its lack of genotoxic effect could become significant.

One of the other striking effects of the experiment described in Chapter 3 of this thesis is the significant increase in the colonic DNA damage induced by high consumption of soy. This occurred despite any reduction in the thickness of the colonic mucus layer as found with other protein types. The data regarding soy consumption and CRC risk are contradictory. A previous rodent study showed that soy-fed rats showed lesser tumour formation compared to those fed without soy (252). It was suggested that minor components (eg isoflavones and saponins) may be responsible for the attenuation of the cancer formation by inhibiting proliferation (253). Furthermore, previous studies suggest that antioxidant properties of soy isoflavones may prevent breast cancer (254, 255). In addition, Mitchell and Collins (1999) (256) demonstrated using the comet assay that soy supplements decrease DNA damage in human lymphocytes. However, other studies indicate that consumption of dietary soy increases damage to epithelial cells in the large intestine (155, 156). Furthermore, soy saponins are known to increase bile acid and steroid excretion and secondary bile acids are cytotoxic within the large bowel (257-259). In addition, soy also increases faecal fat and this may contribute to the increase in damage (156). Whatever the other reported effects of soy, the current data confirm that DNA damage was induced by a non-animal protein source and that the capacity of individual proteins to induce strand breaks needs to be examined on a case-by-case basis.

Chapter 5 of this thesis compared the effects of dietary red and white meat on colonic DNA damage and other indices of bowel health. The rats consuming red meat caused a significantly greater number of colonic DNA SSB and DSB than white meat.

These findings are consistent with epidemiological data (89-91, 117, 260). Red meat contains greater amounts of heme in comparison to white meat and heme is known to stimulate production of genotoxic endogenous N-nitrosocompounds (NOC) in the human gut (129, 131). Hence differences in heme might explain the observed differences in DNA damage between the meat sources. Furthermore, endogenous N-nitrosation can lead to formation of promutagenic and toxic DNA adducts such as O^6-carboxymethyl guanine (58).

In conclusion, this thesis demonstrated that different protein types have differential effects on the integrity of colonocyte DNA. However, further investigation is necessary to identify the mechanisms by which different protein types affect the integrity of colonocyte DNA.

6.1.2 Protective effects of RS

The role of dietary fibre in CRC is not clear-cut. Several epidemiological studies showed that dietary fibre intake is negatively associated with CRC risk (78, 81, 140). These include a large European multi-centre prospective study which showed a dose-dependent reduction in incidence with greater fibre intake. However, other studies have failed to show any protective effects. An early meta-analysis showed that RS, and not fibre NSP, contributes to protection against CRC (100). RS is highly fermentable in the large bowel and leads to SCFA production rather more than NSP (74). The studies in this thesis demonstrate that RS consumption reduces colonic DNA damage, increases SCFA production in the colon and restores the thickness of the colonic mucus barrier. This occurred in the presence of wheat bran as an NSP source.

One of the basic hypotheses in this thesis was that higher large bowel SCFA levels (produced from RS fermentation) correlated with greater protection against protein-induced DNA damage. As expected from the large body of literature, greater RS consumption led to overall increases in caecal and faecal SCFA. The majority of the studies showed that total and individual SCFA pools correlated with colonic mucus layer thickness and inversely correlated with colonic DNA damage for both SSB and DSB. Furthermore, of all SCFA, butyrate showed the strongest correlation with colonic DNA damage. This finding supports many previous studies which show that butyrate is the key SCFA for maintenance of large bowel health (74, 261-263). The precise mechanism whereby this protection occurs needs further study, especially in the context of the mechanism whereby dietary proteins induce that damage.

It needs to be recognised that dietary RS (and fibre) may protect against CRC by mechanisms other than SCFA production. One of the most widely discussed is one which was proposed several decades ago by Dennis Burkitt (195) and suggested that the greatest benefit of fibre was through faecal bulking which diluted luminal toxic compounds and minimised contact between them and the colonic wall. The studies in this thesis support this hypothesis with strong inverse correlations between caecal bulk and colonic DNA damage. As indicated, greater SCFA production is a further mechanism of protection. The data presented in Chapter 4 of this thesis demonstrate that SCFA, especially butyrate, showed strong inverse correlation to colonocyte DNA damage. SCFA are thought to have multiple effects on colonic epithelial cells at different stages in growth, development, transformation and cell death that may explain a decrease in cancer risk (264). Although SCFA have a trophic effect on the normal colon, they seem to exert opposite effects on cancer cell lines by increasing apoptosis and decreasing proliferation (265). Butyrate is a

preferred metabolic fuel for normal colonic epithelial cells but inhibits proliferation and stimulates differentiation of human colon cancer cell lines (204, 205, 265, 266). Moreover, *in vivo* studies have shown that butyrate influences colonic mucus secretion (267-269). The studies in this thesis demonstrated a strong correlation between caecal butyrate and the colonic mucosal barrier. In addition, RS consumption increased overall apoptosis levels of colonocytes, which supports the findings of a previous study (270).

6.1.2.1 Dose-Response Effects of RS

One of the main criticisms of the studies conducted in chapters 2 and 3 was the high content of RS in the diet (48% HAMS) which is rather higher than that considered to be representative of human intakes. Chapter 4 of this thesis tackled this issue by conducting a study on various levels of RS on a high protein diet to determine the effectiveness of RS at lower dosages. The results showed for the first time that the reduction in comet tail moment by increased dietary RS was a dose-dependent one. Furthermore, it was found that even an inclusion of 10% HAMS significantly increased colonic butyrate production and was able to significantly reduce the high protein induced colonic DNA damage. A previous dietary study suggests this dose is achievable in a human diet (271).

6.1.2.2 Oxidative Damage

Another potential protective mechanism investigated in this thesis was oxidative damage. Oxidative damage through free radical generation was proposed as a potential contributor to experimental carcinogenesis by the heme iron in red meat (247, 272). It has

been shown in an *in vitro* (cellular) study that both acetate and butyrate reduced oxidative damage but isobutyrate and propionate were ineffective (273). However, there appear to have been no previous studies of the effectiveness of RS in reducing oxidative damage. In chapter 4, the endonuclease III FLARE comet assay was used to determine whether dietary RS induces changes in oxidative damage. Subtraction of the baseline values from those with endonuclease III showed that the contribution of 8-hydroguanosine formation was small and that tail moment was independent of dietary RS level. Therefore, it appears that RS does not lower colonic DNA damage through lowering oxidative damage. This leaves other potential protective mechanisms of RS including absorption of carcinogens, modification of intestinal microflora, alteration of faecal bile salt excretion and lowering of pH (204).

6.1.3 Mucus Layer Thickness

Thickness of the mucus barrier has been identified as a key protective element in the health of the large bowel. Intestinal mucins, secreted by goblet cells, forms a highly hydrated mucus gel coating the epithelial surface of the intestinal tract (274). As mucins play a key protective role to underlying epithelium, any quantitative change in mucus secretion may modify this defensive barrier (275, 276). Furthermore, loss of barrier function is a prominent feature of inflammatory bowel diseases (277). For these reasons, the thickness of the colonic mucus layer was measured in every experiment throughout this thesis. With the exception of soy protein, the combined results indicate that increased DNA damage correlated with thinning of the mucus layer thickness. The reason for this differential effect of soy is unclear. However, in every case where there was mucus

thinning of the mucus layer, it was countered by inclusion of RS in the diet. One possible mechanism for this protection may be increased fermentation and therefore, the production of SCFA. Previous investigations suggested that dietary supplementation with fibre may modify the mucin composition favourably or increase the number of goblet cells (278, 279). Furthermore, *in vivo* studies indicate that SCFA increase colonic mucin secretion (267-269). Furthermore, a human biopsy study showed butyrate in particular reduced sulphide induced mucosal hyper-proliferation (280). SCFA may inhibit colonic mucin fermentation by reducing sulphide production while enhancing mucin secretion. The interactions between dietary resistant starch and secretory activity of goblet cells may provide a means for the manipulation of the mucus barrier to improve its protective functions.

6.1.4 Red Meat and DNA DSB

The experiments described in chapter 5 revisited the effect of red meat on CRC risk. This time consumption of red meat was compared to that of white meat and their dose-dependencies (15, 25 and 35% protein contents) in terms of colonic DNA damage. Both DSB and SSB were measured in colonocytes. DSB is a more potent form of DNA damage compared to SSB and is considered to be a better indicator of CRC risk. The results demonstrated that increases in dietary red meat led to increased DSB as well as SSB. Furthermore, this increase in DSB was seen only with dietary red meat consumption and not white meat. DSB can result in chromosomal aberrations as they can affect many genes (52) leading to the malfunctioning of cells and cell death. Furthermore, several studies have shown a strong link between impaired DSB repair systems and predisposition

to cancer (281, 282). The results of this study suggest strongly that red meat consumption changes the integrity of colonocyte DNA more than white meat and that similar changes in humans could be a contributing factor to the differential risks of red and white meat consumption on CRC as indicated by epidemiological studies (87, 89, 91).

In summary, this work has demonstrated that the consumption of high protein diets (as casein, soy and red meat) increased colonic DNA damage. The effects of red meat support the findings of the previous studies (89, 90, 107, 140). The studies demonstrated that there were differential effects of protein induced colonic DNA damage depending on the types of proteins. In addition, there was a strong correlation between the colonic DNA damage and the colonic mucus barrier thickness and the caecal SCFA production.

6.2 Future Directions

Future research investigating the effect of dietary protein on CRC risk needs to focus on the specific mechanisms involved and how these findings relate to human large bowel health.

6.2.1 Animal and Human Models

The research conducted in this thesis indicates a strong link between increased consumption of several types of protein consumption and increased DNA damage in the colon. Further investigations in other animals (i.e. pigs) which resemble humans more

closely are needed to confirm the findings of this thesis. Rodents are valuable for genotoxicity testing since rodents and humans have similar biological functions. However, rodents and humans have many differences including, lifespan, body weight, gut microflora and gene regulations. A meta-analysis analysed the suitability of rodent models of carcinogenesis in predicting efficacy in humans using aspirin, β-carotene, calcium and wheat bran (283). The results indicated that, for studies on carcinogen induction of tumours, rats matched humans for aspirin, β-carotene, calcium and wheat bran. This indicates that rodents can be used to predict effects in humans. However, one of the disadvantages for using a rodent model in gastrointestinal research is the considerably greater relative size of the rat caecum in proportion to that of humans. Pigs are generally considered as a better model for large bowel study than rodents, due to their comparable size and structure to the human colon (284). However, pigs are significantly more expensive than rats and so tend to be used only after preliminary rodent studies.

There are several hurdles to overcome in order to conduct successful clinical studies on high dietary protein and RS consumption on large bowel health. The main concern for a clinical study is that human colonocytes are very difficult to obtain (285-287). Colonoscopy is one of the few techniques which can be used to obtain intact colonocytes. However, the procedures are invasive with the attendant risk of major complications and can be costly (285, 287, 288). Furthermore, isolating colonocytes from mucus and colon tissues from colonoscopy samples can be challenging. An alternative method for obtaining colonocytes is to collect exfoliated cells from faeces. Lewin *et al.* (57) showed that it is possible to collect exfoliated cells from the colon and measure the damage of the cells. However, the main concern with this method is that majority of exfoliated cells are generally not viable and this means that genotoxicity assays, such as

the comet or micronucleus assays, may not be sufficiently robust to measure genetic damage in these cells. Recently, instead of collecting exfoliated cells, DNA has been isolated from faecal samples to measure chromosomal instability and mutations (234). This method requires a large-volume faecal sample and the genetic mutation sites are detected and quantified with real time polymerase chain reaction (233, 234). However, sensitivities for detecting adenoma and adenocarcinoma are still significantly weaker than those obtained with colonoscopy (289). Nevertheless, faecal DNA biomarkers for cancer screening and early detection tools for large populations look promising.

6.2.2 Mechanisms of Protein Induced Colonic DNA Damage

This research project has demonstrated that there are differential effects on protein induced colonic DNA damage depending on the protein type utilised. It was originally hypothesised that increases in colonic DNA damage were the effect of protein *per se*. However, the findings of the chapter 3 of this thesis indicated that the protein types can influence the extent of colonic DNA damage. Furthermore, it was shown that red meat significantly increased DSB as well as SSB when compared to corresponding white meat protein concentration in the absence of RS. All of the studies conducted in this thesis suggest that the mucus layer thickness correlated with colonic DNA damage. The sole exception was soy, for reasons which are unclear. These results suggest that genotoxins may reduce the thickness of the mucus barrier by influencing the turnover rate or the production rate of mucus by epithelial cells. Hence, it is likely that the mechanisms of protein induced colonic DNA damage on the large bowel health may be more complex than previously anticipated.

This thesis focused on DNA damage as single and double strand DNA breaks. However, other types of DNA damage may be involved eg base loss, chemical modifications, replication errors and cross link formation. The comet assay was used to measure the DNA damage of the colonocytes, but there are some limitations attached to this method. The main one is that interpretation of the data can be complicated by the fact that there is no simple relationship between the amount of DNA damage caused by a specific chemical and its biological impact (239, 290). Furthermore, if the samples contain predominantly necrotic and apoptotic cells, accurate information cannot be obtained (239). Further studies are needed to clarify the mechanisms of how certain protein sources increase colonic DNA damage.

6.2.3 Protective Mechanisms of RS

Whether the RS protects large bowel health via faecal bulking effects or increased SCFA production is yet to be determined definitively. Since the majority of SCFA are produced during the fermentation process, it is difficult to determine whether faecal bulk or SCFA production provides large bowel protection. Furthermore, it is most likely that faecal bulking and SCFA production protect the colon at a different stage of carcinogenesis. Faecal bulking will dilute toxins and carcinogens in the faeces and reduce intestinal transit time, thus minimising mucosal exposure to potential carcinogens or tumour promoters (74, 165, 166). However, it is unlikely that this dilution and reduced transit time are beneficial at adenoma or carcinoma stages of carcinogenesis. In contrast SCFA, butyrate in particular, have been shown to increase apoptosis and reduce proliferation (262, 291). It has been shown that RS consumption increased apoptosis in

carcinogen damaged rat colonocytes (292). Furthermore, SCFA modifies the composition of the microflora, especially by stimulating beneficial bacteria including bifidobacteria and lactobacilli (292-294).

Recent introduction of modified starch sources may clarify the protective effects of RS. These modified starches are acylated with specific SCFA which are released by bacterial action in the colon (295, 296). Therefore, these starches will be able to determine the effects of SCFA on colonic DNA damage independently of faecal bulking and of fermentation. The complexity of the large bowel system makes identification of the specific mechanisms difficult. Colonic SCFA production is known to change the bacterial populations and there are still large numbers of micro-organisms in the gut which are yet to be identified. It is possible that RS alters cancer risk by modifying bacterial metabolism (eg carcinogen production) by subtle changes which need not involve SCFA. .

6.2.4 Red Meat and Colorectal Cancer Risk

The dose-dependent increases in both DNA SSB and DSB with dietary red meat support the idea that this protein source has adverse effects on the integrity of colonocyte DNA. One of the common explanations for the association between increase risk of CRC and red meat consumption is increased formation of heterocyclic amines (HCA) while meat is cooked. The 4th chapter of this thesis cooked both red meat and white meat by the same procedure to minimise carcinogenic compounds linked to different cooking procedures and still showed significant differences in colonic DNA damage. Red and processed meat contains greater amounts of heme in comparison to poultry (129). Heme is known to stimulate the production of genotoxic endogenous NOC in the human

gastrointestinal system (131). A human study showed that the high levels of red meat consumption (600 g a day) had the same magnitude of concentration of total NOC as cigarette smoke (129). Moreover, endogenous N-nitrosation can lead to the formation of promutagenic and toxic DNA adducts such as O^6-carboxymethyl guanine (58). Another possible mechanism for the red meat induced colonic DNA damage is the formation of hydrogen sulphide during bacterial metabolism of dietary proteins. Carbohydrates are the preferred substrate for most colonic bacteria compared with protein. In the absence of dietary carbohydrates, colonic bacteria may degrade host mucin by formation of hydrogen sulphide (297). Red meat is known to be high in sulphur amino acids and a human study has shown that red meat consumption dose-dependently increases faecal sulphide (298). Moreover, recent evidence indicates that increased protein fermentation as a consequence of a high protein diet can produce harmful compounds such as phenol, cresol, indoles, amines and ammonia (104). High concentrations of these compounds are toxic to the epithelium and may promote genomic instability. A recent rodent study of AOM-induced tumours demonstrated that undigested protein promoted adenocarcinoma in the small intestine (299). However, these toxic by-products of protein metabolism were reduced when RS was added to the diet (116, 300). The studies in this thesis support this and showed significant reduction of phenol and *p*-cresol, which are implicated in bowel cancer (116), in both caecum and faeces with RS diets. Accumulation of these harmful by-products of protein metabolism may be reduced by the fermentation of carbohydrate. Moreover, lowering of colonic pH through acidification via increased SCFA may limit the production of ammonia. Further investigations are necessary to determine how increased DNA damage caused by red meat is associated with increased risk of CRC.

Finally, this thesis has contributed some new information on the interactive effects of high protein diets and RS *in vivo*. High dietary protein, red meat in particular, appears to be harmful to the health of the large bowel, but addition of RS to the diet protects against this damage possibly via SCFA production and the maintenance of the colonic mucus layer thickness. One of the unanswered questions is the absence of a substantial genotoxic effect of whey and the relatively smaller effect of white meat. The fact that soy induced SSB without loss of mucus barrier also needs to be investigated. The potential for RS to be effective in maintenance of the large bowel health is also of considerable interest and this and its interaction with dietary protein is an area needing more research.

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