THE EFFECT OF DAIRY AND RED MEAT ON METABOLISM AND COLON 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.

I give my consent to this copy of my thesis, when deposited in the University library, being available for photocopy or loan.

SIGNED .... DATED...7/4/04........

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ABSTRACT

This thesis describes a series of experiments that aimed to investigate the effects of dairy and red meat on metabolism and colon health. There were four main experimental directions followed.

Effect of fat type and heme on risk markers of colon cancer

Heme iron is a component of red meat that has been shown to effect biomarkers associated with colon cancer risk. It was not clear whether different dietary fat types may interact with heme to influence fecal water cytotoxicity or colon proliferation. Rats were fed diets containing 20% fat as sunflower seed oil (SSO) or milk fat (MF) and heme iron was added to these diets at 0, 0.02 and 0.08g/100g. Addition of heme to the SSO based diet increased cecal and fecal water cytotoxicity, colon crypt height, fecal moisture, fat and cation concentration. These differences were not seen for the MF diets. Thiobarbituric acid reactive substances (TBARS) in the fecal and cecal water and urine were positively related to the concentration of heme in the diet, but were not affected by fat type. The increase in fecal and cecal water cytotoxicity for rats consuming the heme and SSO diets, but not heme and MF diets may be due to the types, rather than concentration of lipid peroxides formed from the different dietary fats.

Heme iron: an initiator of colon cancer?

Heme iron together with polyunsaturated fat has been shown to be genotoxic to cells in vitro. It was hypothesized that feeding a high heme/polyunsaturated fat diet to rats would increase the amount of genetic damage in colonocytes. Colonocytes were isolated and then analysed for DNA damage using the Comet assay. Animals fed the heme/polyunsaturated fat diet showed an increase in the amount of genetic damage in the colonocytes isolated from the distal colon, in comparison to animals fed a control diet. If the heme induced genetic damage occurred to specific genes such as APC, DCC or p53 this may lead to the initiation of cancerous cells. Further investigation is required to determine the role of heme as an initiator of colon cancer.

Dietary protein type and density: effect on colon cancer risk

In the third study, the effect of red meat, whey protein and their density in the diet on aberrant crypt foci (ACF) expression was examined in azoxymethane treated Wistar rats. Increasing red meat density correlated positively with rate of weight gain, while increasing whey protein density correlated negatively. Dietary intake was not affected by protein type or density. The 32% WPC group had significantly less ACF in the proximal colon in comparison to the 16% and 32% red meat groups. This reduction in ACF number in the whey protein group may be caused by hormones associated with the reduction in weight gain, and/or by components of whey protein such as cysteine, lactose and conjugated linoleic acid. These components have been shown to have anti-cancer effects. When using ACF number as an marker of colon cancer risk, whey protein appeared to be more protective than red meat. Increasing the density of red meat in the diet did not increase ACF expression.

Dietary protein type and density: effect on metabolism

Studies in growing rats have shown that red meat may increase weight gain where as whey protein may reduce weight gain. It was hypothesized that in mature insulin resistant Wistar rats, increasing the density of whey protein, but not red meat, would reduce weight gain, body fat storage and improve insulin sensitivity. The high protein diets reduced energy intake, visceral, subcutaneous and carcass fat and increased carcass protein. The high protein diets also lowered fasting plasma triglyceride and IGF-I. Increasing the density of whey protein, but not red meat in the diet was associated with a reduction in body weight gain. This is supported by a reduction in plasma insulin and improved insulin sensitivity for the high whey protein group. These findings support the conclusion that the high protein diet was effective in reducing energy intake and adiposity and that WPC was more effective than red meat in reducing body weight gain and improving insulin sensitivity.

Summary

The heme iron content of red meat has been proposed as a possible way that red meat may increase colon cancer risk. In this thesis it was shown that a high heme iron diet in the presence of polyunsaturated fat but not saturated fat (AMF) increased colon cancer risk by increasing the cytotoxicity and genotoxicity of fecal water. However, when a high red meat and polyunsaturated fat diet was fed to rats it did not increase ACF expression in comparison to a low red meat diet. The lack of an effect may be due the increased absorption of heme from red meat which leaves insufficient heme in the colon to increase colon cancer risk. This research suggests that heme iron in a high red meat diet is not likely to be a contributing factor to increase colon cancer risk.

In comparison to red meat, whey protein may be an alternative protein source that could be incorporated into the diet to protect against colon cancer risk. The high whey protein fed animals had significantly less ACF in the proximal colon in comparison to the moderate and high red meat fed animals. A possible mechanism whereby whey protein may inhibit colon carcinogenesis is by improving insulin sensitivity through reducing weight gain and body fat deposition. The added benefit of whey protein is that it may be an effective dietary factor to assist reducing the risk of NIDDM, insulin resistance or obesity. In addition, whey protein may also improve disease management for individuals who have either one or more of the conditions.

PUBLICATIONS ARISING FROM THESIS

Belobrajdic, DP, McIntosh, GH, Owens, JA. A high protein diet provided by whey protein concentrate reduced body weight gain and alters insulin sensitivity relative to red meat in Wistar rats. Journal of Nutrition, in press.

Belobrajdic, DP, McIntosh, GH, Owens, JA. Whey proteins protect more than red meat against azoxymethane induced ACF in Wistar rats. Cancer Letters, 198, 43-51, 2003.

Belobrajdic, DP, McIntosh, GH, Owens, JA. The effects of dietary protein on rat growth, body composition and insulin sensitivity. The Australian Journal of Dairy Technology, 58 (2), 176, 2003.

Belobrajdic, DP, McIntosh, GH, Owens, JA. Protein quality and quantity influence the expression of aberrant crypt foci (ACF) in Wistar rats. Journal of Gastroenterology and Hepatology 17 (Suppl.), A172, 2002.

PRESENTATIONS

Belobrajdic DP, McIntosh GH and Owens JA. The effect of dietary protein on rat growth, body composition and insulin sensitivity. Presented at Nutrition Society, Hobart, Australia, 30th November – 3rd December, 2003.

Belobrajdic DP, McIntosh GH and Owens JA. The effect of dietary protein on rat growth, body composition and insulin sensitivity. Presented at the ADS/ADEA Annual Scientific Meeting in Melbourne, Australia, 17th – 19th September, 2003.

Belobrajdic DP, McIntosh GH and Owens JA. The effect of dietary protein on rat growth, body composition and insulin sensitivity. Presented at the IDF 2nd World Symposium of Dairy Products in Human Health and Nutrition, Melbourne, Australia, 24-27th August, 2003. **Awarded best poster in the student category.**

Belobrajdic DP, McIntosh GH and Owens JA. The effect of dietary protein on rat growth, body composition and insulin sensitivity. Presented at the Australian Society for Medical Research (SA) Scientific Conference, Adelaide, Australia, 30th May, 2003. **Awarded Holden Young Investigators Award for best presentation.**

Belobrajdic DP, McIntosh GH and Owens JA. The effect of dietary protein on rat growth, body composition and insulin sensitivity. Presented at Congrilait (World Dairy Congress), Paris, France, 24-27th October 17, 2002.

Belobrajdic DP, McIntosh GH and Owens JA. Protein quantity and quality influence the expression of aberrant crypt foci (ACF) in Wistar rats. Presented at the Australian Gastroenterology Week, Adelaide, Australia, 15-18th October, 2002.

McIntosh, GH and **Belobrajdic DP.** Dairy proteins – their role in human health and cancer prevention. Presented at the Australian Institute of Food Science and Technology (AIFST) Conference, Sydney, Australia, 23rd July 2002.

Belobrajdic DP, McIntosh GH and Owens JA. Influence of dietary fats and heme on risk markers for colon cancer. Presented at the Australian Society for Medical Research, South Australian Division, Adelaide, Australia, 30th May, 2002.

ABBREVIATIONS

ACF	aberrant crypt foci
APC	adenomatous pholyposis coli
AIN	American Institute of Nutrition
AOM	Azoxymethane
BMI	body mass index
BW	body weight
COX-2	cyclooxygenase 2
CYP	cytochrome P450
°C	degrees, Celsius
DCC	deleted in colorectal cancer
DAG	diacylglycerol
DMBA	dimethylbenz(a)anthracene
DMH	dimethylhydrazine
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
DTT	dithiothreitol
EDTA	ethylenediamine-N,N,N,N-tetra-acetic acid
FFA	free fatty acid
g	grams
g	force of gravity
GSH	glutathione
GST	glutathione S transferase
h	hours
HBSS	Hanks buffered salt solution
HCA	heterocyclic amines
4-HNE	4-hydroxy-2-nonenal
IGF	insulin like growth factor
IGF-I	insulin like growth factor I
IGF-BP	insulin like growth factor binding protein
kJ	kilojoules

kg	kilograms
LC-CoA	long chain co-enzyme A
MDA-DNA	malondialdehyde deoxyribonucleic acid
MTHFR	methylene tetra hydro folate reductase
µg	micrograms
µl	microlitres
mg	milligrams
mm	millimeters
mOsM	milli-osmolar
min	minutes
MAP kinase	mitogen activated pathway
NAT	N-acetyl transferases
NIDDM	non-insulin-dependant diabetes mellitus
nm	nanometers
NOC	N-nitroso-compounds
ODC	ornithine decarboxylase
PPARS	peroxisome proliferators activated receptors
PL	phosopholipids
PA	phosphatidic acid
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PKC	protein kniase C
R	alkyl radical
ROS	reactive oxygen species
ROO	lipid alkyl peroxyl radical
ROOH	lipid hydroperoxide
RPMI	Rosewell Park Memorial Institute medium
SSO	sunflower seed oil
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
TBARS	thiobarbituric acid reactive substance
TG	triglyceride

TBS	tris buffered saline
V	volts
VLDL	very low density lipoprotein
w/w	weight/weight

Chapter 1 - INTRODUCTION

1.1 Colorectal cancer

1.1.1 Epidemiology

Colon cancer is the second most common cause of death from cancer in the Western world. In Australia, colorectal cancer was the most common cancer diagnosed in 1999, with 11,637 new cases and was the second leading type of cancer causing death (4,575 cases), second only to skin cancer (AACR, 2002). Worldwide there is a 20-fold variance in colon cancer incidence rates (Potter, 1996). Western countries including America, Europe and Australia have the highest incidence of colon cancer, accounting for nearly two thirds of worldwide incidence. Population based epidemiological studies have shown that when individuals migrate from low-risk to high-risk areas, risk for colon cancer increases accordingly to match the host population (Bingham et al, 1996). Although it is recognized that genetic factors are important determinants in the development of colorectal cancer in individuals, environmental factors, such as diet composition, may account for up to 70% of colon cancer causation (Doll and Peto, 1981; Willett, 1995).

1.1.2 Pathogenesis of colon cancer

Colorectal carcinogenesis is a complex, multistep process involving both genetic and environmental factors. Cancer is thought to be caused by an accumulation of multiple genetic mutations resulting in a transformed phenotype and eventual progression and invasion of other tissues and organs in the body (**Figure 1.1**) (Vogelstein et al, 1988).

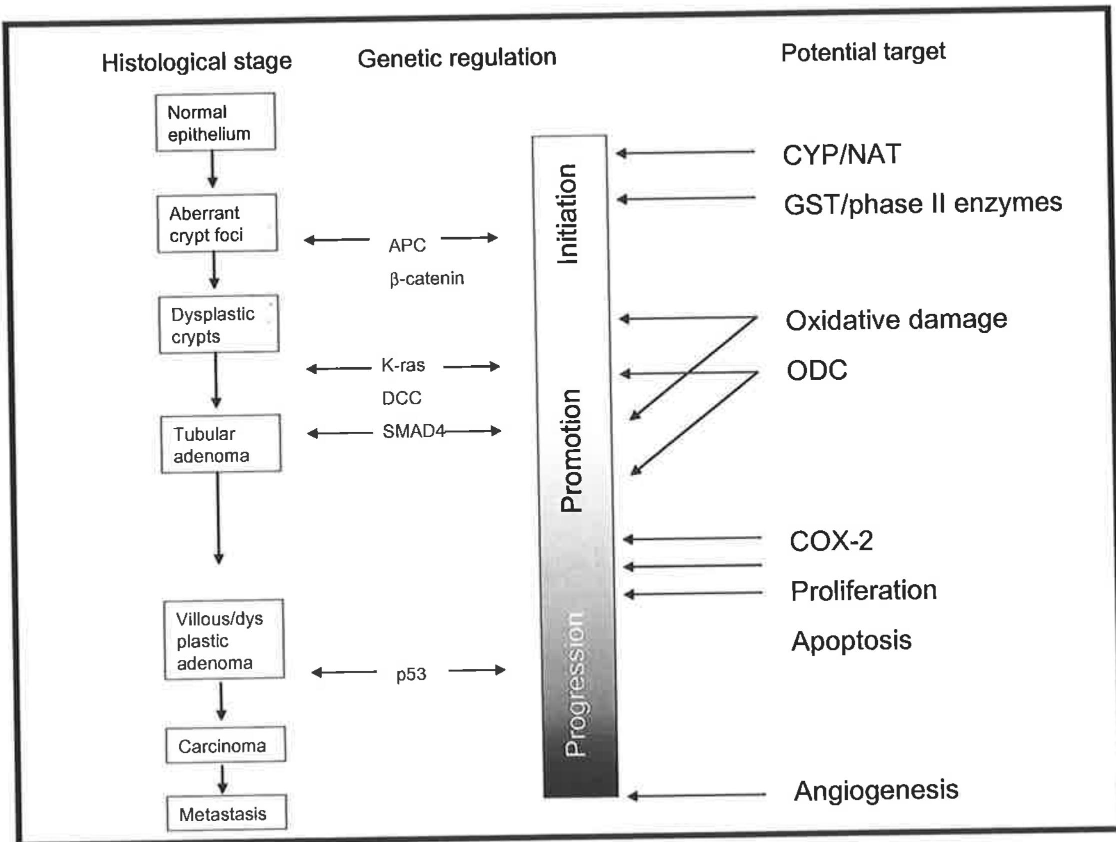


Figure 1.1 Multistep model of carcinogenesis with targets for chemoprevention. Significant genes involved in regulation at sequential stages in carcinogenesis include adenomatous polyposis coli (APC), K-ras, DCC and p53. A number of inducible enzymes are involved at critical stages in promotion and/or protection. APC, adenomatous polyposis coli, COX-2, cyclooxygenase; CYP, cytochromes P450; DCC, deleted in colorectal cancer; GST, glutathione S-transferase; NAT, N-acetyl transferases; ODC, ornithine decarboxylase. Modified from Sharma et al (Sharma et al, 2001).

Initiation, the first stage of carcinogenesis, is associated with mutations in the adenomatous polyposis coli (APC) and β-catenin genes (**Figure 1.1**). Both these genes play a pivotal role in the regulation of mucosal proliferation and are therefore classical tumor suppressor or gatekeeper genes. There are two possible processes that may be responsible for inducing genetic mutations in colonocytes, including either 1) the direct action of a mutagen causing genetic damage or 2) increased proliferation causing an increased risk for an error occurring.

A number of different agents have been identified to cause genetic damage to colonocytes. Some of the known agents include chemical mutagens, dietary contaminants, irradiation, pathogenic bacteria and viruses. Mutations may not necessarily lead to the development of cancer as cells with genetic muations may be removed by the process of apoptosis (programmed cell death). Often other factors are needed to create a sympathetic environment for the progression of the mutated gene.

There are many non-genotoxic agents that are associated with increased risk of colon cancer. These agents include hormones, drugs, infectious agents, chemicals, physical or mechanical trauma and other chronic irritations (Preston-Martin et al, 1990). A common histological change induced by these agents is an increase in proliferation. Each time a cell divides there is a small chance that a genetic error related to the carcinogenic process will occur. When these agents produce a sustained increased in cell proliferation they enhance the likelihood of cancer development by providing additional cell divisions, each with an opportunity for spontaneous genetic error (Cohen et al, 1991).

Following initiation, aberrant crypt foci (ACF) development is the earliest stage where morphological changes can be identified in the colonic epithelium. Morphological changes that are characteristic of an ACF include enlarged epithelial cell wall and diameter, slit like lumen, loss of goblet cells and elevation from the crypt surface (**Figure 1.2**). ACF commonly show histological changes that include increased cell proliferation, dysplasia, altered histo-chemical state and altered mucin secretion (Pretlow et al, 1994; Yamashita et al, 1994). Furthermore, ACF often have genetic changes in key genes (APC tumor suppressor gene, K-ras and c-fos oncogenes) which are related to the progression of colon carcinogenesis to crypt dysplasia and adenoma formation (**Figure 1.1**) (Stopera and Bird, 1992; Stopera et al, 1992; Vivona et al, 1993).

The second stage of carcinogenesis, promotion, is commonly associated with mutations in K-ras, DCC and SMAD4 (**Figure 1.1**). Pathological changes associated with these mutations include dysplasia of crypt cells and the formation of tubular adenoma. Factors that were involved in inducing genetic mutations in the initiation stage can also be involved in the promotion stage. During promotion there is a marked increase in the proliferation rate of epithelial cells which may further increase the risk of mutations occurring in the DNA (Deschner, 1982; Terpstra et al, 1987).

The final stage of carcinogenesis, progression, involves additional growth of the tubular adenoma and invasion of the basement membrane (**Figure 1.1**). Loss of the tumor suppressor gene, p53 is the major genetic change associated with progression of tumor growth to carcinoma. The p53 gene is particularly important in arresting cell cycle

progression, thus allowing the DNA to be repaired; or it increases apoptosis. These functions are achieved, in part, by the transactivational properties of p53, i.e. it activates a series of genes involved in cell cycle regulation. In cancer cells bearing a mutant p53, there is no longer control of cell proliferation, resulting in inefficient DNA repair and the emergence of genetically unstable cells (Soussi, 2000).

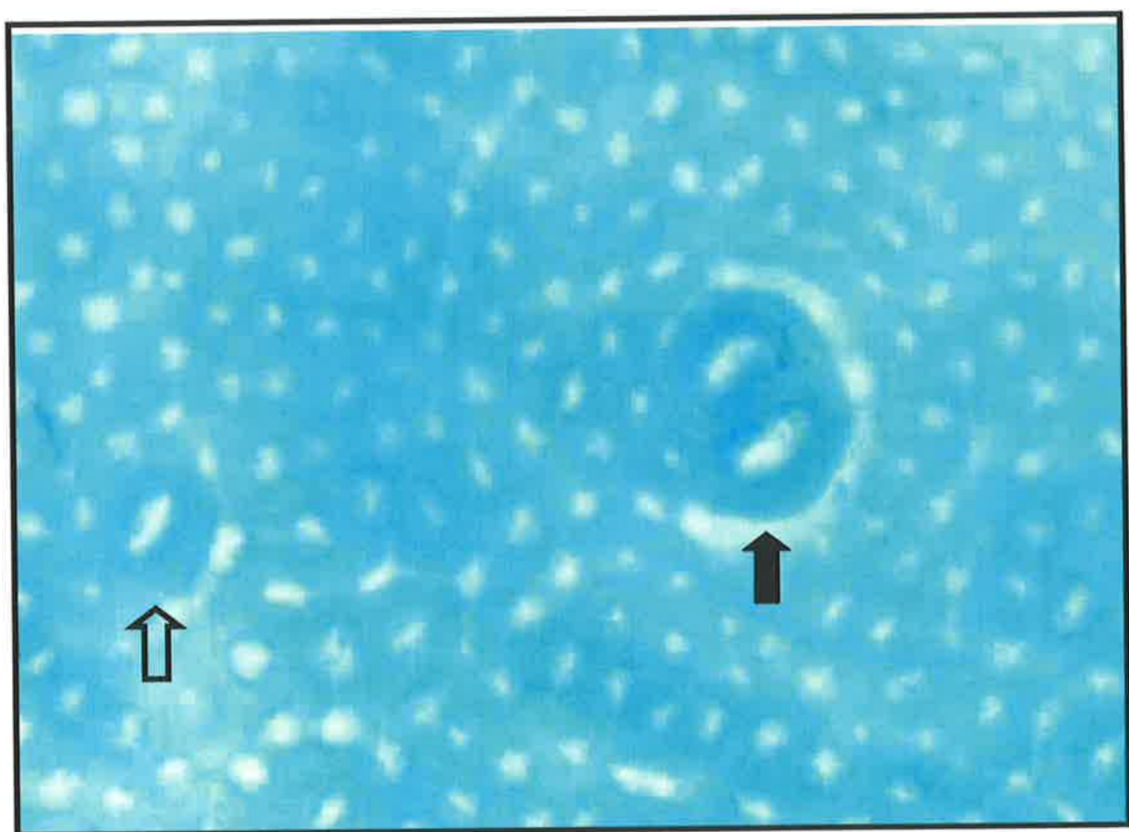


Figure 1.2 En face section of distal colon from a rat administered with azoxymethane. 40 x magnification, section stained with 0.2% methylene blue. Open arrow, single aberrant crypt foci (ACF). Filled arrow shows two aberrant crypts per focus.

1.2 Western diet, insulin resistance and colon cancer risk

High dietary intakes of total fat, animal fat, red meat and low dietary intake of vegetables, typical of the Western diet, have been related to an high incidence of sporadic cancers (colon, breast and prostate) (WCRF, 1997). In addition, these dietary factors have also been associated with an increased risk of developing insulin resistance (Hauner, 2002). This suggests that components of the Western diet may be increasing colon cancer risk through metabolic and signaling pathways involved in insulin resistance.

1.2.1 Insulin resistance

In insulin resistance, the body becomes insensitive to insulin action, thus compromising peripheral insulin-stimulated glucose disposal, particularly by skeletal muscle, and also impairs insulin inhibition of hepatic glucose production. Pancreatic beta cells respond by increasing insulin secretion (hyperinsulinemia) to maintain glucose homeostasis. The abundance of free fatty acids (FFA) (derived from a meal, or high circulating levels in plasma among obese individuals) also significantly affect the degree to which cells utilize glucose as high concentrations of FFA inhibits the utilization of glucose by the cell (**Figure 1.3**).

The utilization of glucose by a cell begins with facilitated diffusion of glucose into the cell, mediated by one or more GLUT transporters. Following uptake, glucose is phosphorylated to glucose 6-phosphate, which can then be stored as glycogen or undergo glycolytic degredation to pyruvate, conversion to acetyl-CoA and citrate where it can be utilized by the Krebs cycle (Belfiore, 1980). Repletion of Krebs cycle intermediates

causes some citrate to leave the mitochondria and enter the cytosol, where it is converted to acetyl-CoA and malonyl-CoA (Prentki and Corkey, 1996). The latter compound inhibits CPT-1, thus reducing the intramitochondrial transport of long chain CoA (LC-CoA) (and therefore its β-oxidation), which tends to accumulate in the cytosol (McGarry et al, 1977; McGarry et al, 1978; Mills et al, 1983).

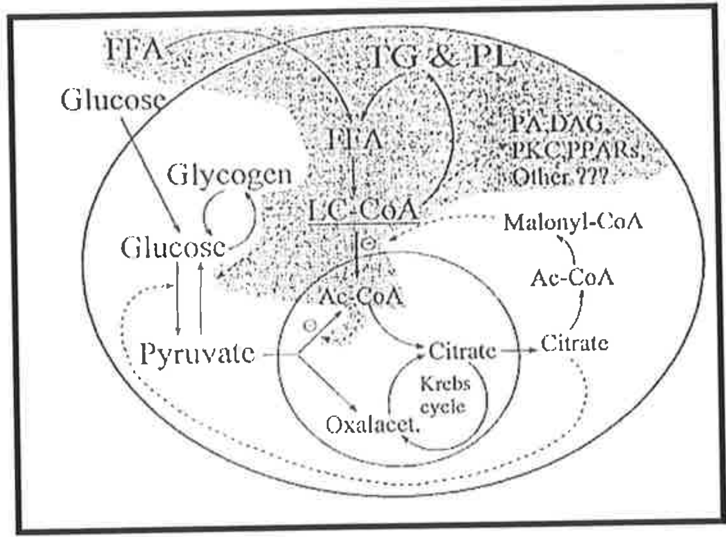


Figure 1.3. Reciprocal inhibitory effects between the metabolism of glucose and FFA.

Glucose metabolism is shown in the white background; free fatty acid metabolism is shown in the gray background. Dotted arrows indicate the regulatory effects. TG, triglyceride; PL, phospholipids; PA, phosphatidic acid; DAG, diacylglycerol; PKC, pyruvate kinase C; PPARs, peroxisome proliferators activated receptors (Belfiore and Iannello, 1998).

In muscle, most of the FFA primarily comes from plasma (in other tissues a significant portion may be derived from intracellular lysis of triglycerides). After entry into the cell FFA is activated to form LC- CoA. LC-CoA is then transported into the mitochondria where it undergoes β-oxidation to form acetyl-CoA, which is finally oxidized in the Krebs cycle (Belfiore, 1980). LC-CoA can also be metabolized in the cytosol where it can exert metabolic and biologic effects. It can be metabolized to form triglyceride, phosphatidic acid and diacylglycerol which are able to regulate the activity of protein kinase C, an important regulatory enzyme (Prentki and Corkey, 1996). Furthermore, LC-CoA can stimulate peroxisome proliferators activated receptors which mediate the induction of several genes encoding enzymes of energetic metabolism (Castelein et al, 1994; Gulick et al, 1994).

When the metabolism of FFA is stimulated following a high fat meal or in obese individuals, cells can utilize both glucose and FFA. Increased entry of FFA into cells enhances the production of LC-CoA in the cytosol and acetyl-CoA in the mitochondria, which leads to the inhibition of glucose utilization, thus inducing insulin resistance. More insulin is released from the pancreas to increase glucose utilization and therefore the production of malonyl-CoA increases which inhibits the β-oxidation of FFA. The net metabolic response in insulin and glucose concentration and FFA utilization varies between individuals (Belfiore and Iannello, 1998). This inter-individual variation in glucose and FFA utilization accounts for the varied levels of insulin resistance in individuals.

In the investigation of the metabolic and other consequent dietary perturbations it is important to use specific measures of insulin action on metabolism which can give insight into whether insulin resistance has been altered. There are many different techniques used to measure insulin resistance. The hyperinsulinemic-euglycemic clamp technique is the gold standard technique to determine the degree of insulin resistance. However, this technique has one draw back in that it induces a persistent hyperinsulinemia that suppresses FFA. This suppression of free fatty acids means that a potentially important metabolic component of insulin resistance in the paradigm understanding may be overlooked. An alternative was developed by Belfiore et al (Belfiore and Iannello, 1998) who devised a formula that calculates insulin sensitivity for glycemia and FFAs based on values of insulin, glucose and FFAs following an oral glucose tolerance test. Formulas have also been devised which allow an assessment of insulin resistance based on fasted plasma insulin and glucose concentrations. These formulas that include, the ratio of insulin to glucose, the homeostasis model assessment and fasting insulin resistance index have all shown strong correlations with the hyperinsulinemic-euglycemic clamp technique (Ahren and Larsson, 2002). Other general markers of insulin resistance include the elevation of plasma triglyceride and the accumulation of adipose tissue and intra-muscular lipid. Any improvements in the concentrations or values of these risk markers indicate that a particular treatment might have improved insulin action.

1.2.2 Insulin resistance and colon cancer risk

Epidemiological evidence supports a link between insulin resistance and increased risk of colon cancer. Firstly, a person with reduced insulin sensitivity, as determined by a raised

plasma insulin and glucose concentration either in a fasted state or following an oral glucose tolerance test has an increased risk of colon cancer (Yamada et al, 1998; Schoen et al, 1999). Secondly, prospective studies have shown that people with Type II diabetes have an increased incidence and mortality rate from colon cancer (Weiderpass et al, 1997; Will et al, 1998; Hu et al, 1999). Thirdly, higher levels of fasting serum triglycerides, a characteristic of the insulin resistance syndrome, is associated with an increased risk of adenomatous polyps and carcinoma (Bird et al, 1996; Yamada et al, 1998). Fourthly, insulin increases the bioactivity of insulin-like-growth factor I (IGF-I), by enhancing its synthesis and by decreasing several of its binding proteins (Giovannucci, 2001; Kaaks and Lukanova, 2001). Increased IGF-I activity is associated with increased risk of colon cancer (Ma et al, 1999; Manousos et al, 1999). Fifthly, elevated plasma concentration of FFA and glucose have been shown to increase the formation of reactive oxygen species (Paolisso and Giugliano, 1996). Individuals with diabetes have an increased production of reactive oxygen species (ROS) and greater oxidative damage to DNA which is related to an increased risk of carcinogenesis (Dandona et al, 1996; Loft and Poulsen, 1996).

The elevated circulating concentration of insulin may be one of the main contributing factors to increased colon cancer risk. Animal studies have shown that growth promoting effects of exogenous insulin, diet induced hyperinsulinemia and hypertriglyceridemia increased AOM-induced colon tumor and ACF expression (Tran et al, 1996; Corpet et al, 1997; Koohestani et al, 1997). Furthermore, in the obese Zucker rat which is predisposed to developing insulin resistance, significantly more ACF developed than in the lean

control animals when rats were administered with a carcinogen (Weber et al, 2000). As these rats were fed *ab libitum* it was not possible to determine whether the promotion of ACF was due to insulin resistance or increased energy intake. Hirose et al (Hirose et al, 2003) recently showed that in db/db mice with an obese and diabetic phenotype, injected with AOM, increased the expression of atypical glandular lesions in comparison to db/+ or WT mice. This elevated expression of atypical glandular lesions in the colon was related to increased insulin and leptin concentration in the serum.

There are currently three major hypotheses suggested regarding the possible role of insulin in colon carcinogenesis; the energy hypothesis, the ROS hypothesis and the IGF hypothesis.

1.2.2.1 Energy hypothesis

In this model it is hypothesized that consumption of excess dietary energy results in the development of insulin resistance, which is characterized by increased circulating levels of insulin, triglycerides and FFAs. These circulating factors may, in turn, initiate a general proliferative response from colonic epithelial cells and promote colorectal carcinogenesis (Bruce et al, 2000).

1.2.2.2 ROS hypothesis

An increased production of ROS in individuals with diabetes may be due either to hyperinsulinemia or hyperglycemia (Bruce et al, 2000). Specific pathways associated with hyperglycemia (glucose autoxidation, polyol pathway, prostanoid synthesis, protein

glycation) are suggested to increase the production of free radicals (Paolisso and Giugliano, 1996). Hyperinsulinemia may also enhance oxidative stress by altering the activity of antioxidant enzymes and free radical generation (Facchini et al, 2000). The resulting production of ROS from hyperglycemia and/or hyperinsulinemia may increase colon cancer risk by the induction of DNA oxidation products (Draper et al, 1995), activating MAP kinase and by increasing the expression of oncogenes such as c-fos and c-jun (Wei, 1992).

1.2.2.3 IGF hypothesis

It has been well established that insulin regulates the IGF system and in particular the concentration of IGF-I, and its binding proteins (**Figure 1.4**). This is of particular interest as IGF-I is a potent mediator of cell survival and growth in the etiology of colon cancer (Ma et al, 1999; Manousos et al, 1999; Khandwala et al, 2000). This has lead to the hypothesis that elevated concentrations of IGF-I may enhance cell growth and proliferation, reduce apoptosis, and increase the transformation of normal and mutated cells.

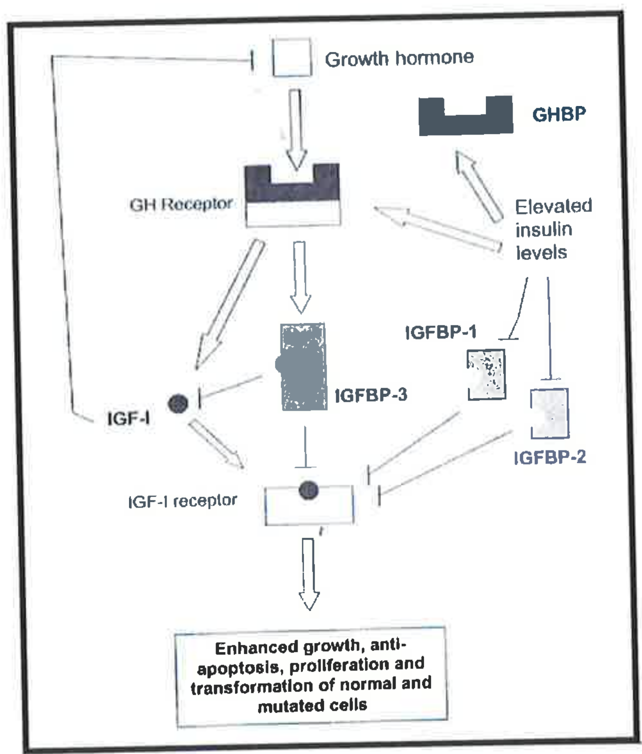


Figure 1.4. Biologic interactions at the pituitary and hepatic levels among insulin, growth hormone (GH), insulin-like growth factor-I (IGF-I), and insulin-like growth factor binding proteins (IGFBPs). Open arrows denote stimulation, and thin black lines denote inhibition (Sandhu et al, 2002).

1.3 Biomarkers of colon cancer

To target the prevention of colon cancer it is necessary to examine the effect of environmental factors, including diet, on biomarkers for colon cancer. Preneoplastic biomarkers are most useful for assessing the efficacy of cancer chemopreventitive agents as they show biological alterations that represent the early and intermediate stages of carcinogenesis (Sharma et al, 2001). There are two major categories of biomarkers for colon cancer research that involve either changes in colonic epithelium or changes in fecal composition.

1.3.1 Biomarkers of the colonic epithelium

Biomarkers that have potential use in colon cancer studies are listed in **Table 1.1**. To assess these biomarkers in a subject, a sample of epithelium is required that can be obtained by pinch biopsy or at autopsy in animal studies. Although this is an invasive technique, a great deal of information can be obtained.

Biomarkers of particular interest to this thesis are the aberrant crypt foci, proliferation and the comet assay. Their usefulness and application are outlined below.

Table 1.1. Biomarkers of colon cancer

Type of biomarker	Variable measured	Biomarker
Pathological	Histology	Adenoma, aberrant crypt foci
	Abnormal mucin expression	ACF stain
Cellular	Proliferation	BrDU, PCNA, Ki67, lectin labeling
	Differentiation	Lectin labeling
	Apoptosis	TUNEL assay, haematoxylin stain
Biochemical	Arachidonate metabolism	Prostaglandins, COX-2, arachidonic ac lipoxygenase, leucotrienes
	Polyamine metabolism	Polyamines, ornithine decarboxylase
	Detoxification enzymes	GST, DT-diaphorase
Molecular	DNA methylation, DNA adducts	Methyl groups, MTHFR,
	Cell cycle	MDA-DNA adducts
	DNA breaks, oxidized pyrimidines,	Cyclin D1, TGFα
	inclusion of AP-sites	Comet assay
Genetic	Gene/product	K-ras, APC, DCC

ACF, aberrant crypt foci; APC, adenomatous polyposis coli; BrDU, bromodeoxyuridine ; COX-2, cyclo-oxygenase; DCC, deleted in colorectal cancer; GST, glutathione S transferase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; MDA, malondialdehyde; MTHFR, methylene hydro folate reductase; PCNA, proliferating cell nuclear antigen; TGFα, transforming growth factor-α

Modified from Sharma et al (2001).

1.3.1.1 Aberrant crypt foci

The ACF is the earliest step in colorectal carcinogenesis and is considered to be a precursor lesion of colonic adenomas and carcinomas (Roncucci et al, 2000). ACF was first identified by Bird (1987) in the colons of carcinogen treated rodents and has been shown to be present in humans who are at risk of developing colon cancer (Pretlow et al, 1992; Roncucci et al, 1998). In rodents, ACF are induced by the administration of a carcinogen. By modifying the diet before, during and/or after exposure to the carcinogen, it is possible to assess the effect of diet on colon cancer risk through altered ACF expression. Biological characteristics of the ACF include; increased cell proliferation, dysplasia, altered histo-chemical state, decrease in mucin and goblet cell content, frequent mutations in ras and c-fos oncogenes and the APC tumor suppressor gene (Stopera and Bird, 1992; Stopera et al, 1992; Vivona et al, 1993; Pretlow et al, 1994; Yamashita et al, 1994). The advantage of this assay is that it allows examination of the early stages of carcinogenesis. ACF are easy to distinguish from the surrounding normal crypts by a light microscope following methylene blue staining. Morphological changes in crypts that are characteristic of an ACF include an enlarged epithelial lining and diameter, slit like lumen, a darker stained appearance and elevation from the crypt surface (**Figure 1.2**).

It must be acknowledged that not all ACF will progress to adenoma or carcinoma. Zhang et al (1992) were the first to suggest that larger ACF (greater than or equal to four aberrant crypts/focus) in the distal colon are more predictive of progression to carcinoma. It has been shown that these large ACF produce a different goblet cell mucin

(sialomucin) which can be identified by histological staining (Caderni et al, 1995; Uchida et al, 1997). Furthermore, the larger crypts that secreted the sialomucin were also shown to be more predictive of dysplasia and thus progression to adenoma or carcinoma (Jenab et al, 2001). Therefore, care must be taken when using ACF as a preneoplastic biomarker for colon cancer. Nevertheless as reported by Corpet and Tache (2002) there is a good correlation between ACF and tumor data in the rat chemical carcinogenesis model.

1.3.1.2 Proliferation/Apoptosis

As mentioned previously (Section 1.1.2), measuring proliferation provides an indication as to how a dietary component or chemical may increase colon cancer risk. Measurement of apoptosis provides valuable information regarding the way in which cells with genetic mutations can be removed. However, in the colon there is a very low rate of apoptosis and meaningful measurements can only be obtained if a chemical carcinogen is administered to increase the rate of apoptosis (Hu et al, 2002).

1.3.1.3 Genotoxicity assay

The Comet assay is a sensitive and rapid technique enabling the detection of DNA strand breaks and alkali-labile sites in individual cells (McKelvey-Martin et al, 1993). It is especially useful as a biomarker of colon cancer as the colonocytes from humans or animals can be used in the Comet assay to assess the degree of genetic damage induced by different dietary or treatment regimes. Alternatively fecal samples from these subjects can be assessed for their potential to induce genetic damage to cells *in vitro*.

1.3.2 Fecal biomarkers

Colonic epithelial biomarkers can be used to test the short or long term effect of the environment or diet on an individual's risk for developing an adenoma or carcinoma in the colon. Further information regarding colon cancer risk can be obtained by assessment of the composition of the fecal contents, which is a less invasive method.

The aqueous phase of feces is often used in examining biomarkers of colon cancer. It is thought that the components of this fecal fraction are more likely to exert damaging effects on the cells of the colonic epithelium than components bound to food residues and the bacterial mass (Haza et al, 2000). The importance of examining the aqueous phase of feces was highlighted in a study where high concentrations of dietary calcium inhibited the metabolism and solubility of heme iron (Sesink et al, 2001). As a result the heme-induced cytotoxic activity of fecal water and colonic epithelial hyperproliferation was prevented (Sesink et al, 2001). Furthermore, dietary changes have been shown to increase fecal water cytotoxicity by increasing the concentration of fecal water bile acids, whist not affecting total fecal bile acid concentration (Rafter et al, 1987).

By studying the aqueous phase of feces it is possible to use fecal water in *in vitro* assays to determine its cytotoxic and genotoxic effects on cultured cells. This information provides insight into whether a particular dietary regime increases the ability of fecal water to exert damaging effects to the colonic epithelium and increase the risk of colon cancer.

1.3.2.1 Cytotoxicity assay

The cytotoxicity assay makes it possible to assess the degree to which the colonic contents may exert damaging effects to the colon epithelium *in vitro*. At present, normal colonocytes are not available for cell culture studies. This is not surprising due to their migratory development from the base to the apex of the crypt, their strong dependence on the basement membrane and hormonal control for development and their short life of approximately four days. Alternatively, cells from colon tumors have been successfully cultured *in vitro* due to their ability to escape normal apoptosis controls. The HT-29 colon cancer cell line is commonly used in the fecal water cytotoxicity assay. It is an adherent epithelial cell line that originated from a human colorectal adenocarcinoma, and has mutant APC and p53 genes.

The erythrocyte has also been used to measure fecal water cytotoxicity. A study by Lapre and van der Meer (1992) showed that exposure of erythrocytes and Caco-2 cells to a fatty acid and mixed micelle produced a very similar degree of cytotoxicity in these two cell types ($r > 0.95$). However, a study by van Munster et al (1993) showed that the HT-29 assay was 10 times more sensitive than the erythrocyte assay in detecting cytotoxicity. In assessing cytotoxicity, the erythrocytes and HT-29 cells may respond differently in comparison to colonocytes due to their differences in cell structure and function. However, in this thesis the HT-29 cell has been chosen due to its many similarities with the colonocyte.

The strong link between fecal water cytotoxicity and colonic proliferation strengthens its use as a biomarker for predicting colon cancer risk. Lapre and van der Meer (1992) observed a highly significant correlation between lytic activity of fecal water (using the erythrocyte) and colonic proliferation ($r = 0.85$, $n = 24$, $p < 0.001$).

1.4 Red meat

Meat and meat products are substantial components of the Western diet. The National Nutrition Survey of Australians (ABS, 1999) showed that the mean intake of red meat for men was 88g and for women 45g (cooked weight of meat, as eaten). Red meat contributed 6% of total energy, 20% of the daily protein, 14% of the iron (52% heme iron), 27% of the zinc and 24% of vitamin B12 intakes but only 8% of total fat intake. This low contribution of meat to total fat intake is a result of two-thirds of the people reporting that the red meat cuts were either trimmed of fat or lean when eaten (Baghurst, 1999).

Although red meat contains components that are important to maintaining a healthy body, such as protein and micronutrients, when consumed in high amounts red meat may increase colon cancer risk. A US Nurses health study reported that the highest quintile of red meat intake (134g per day or more) was associated with increased colon cancer risk (RR=1.77) (Willett et al, 1990). In the Australian survey, 30% of men and 11% of women consumed more than 120g of red meat per day. This is a considerable number of people to be in the US Nurses health study zone of increased risk and may contribute to causing the high incidence of colon cancer in Australia. Components of red meat that

may promote colon cancer risk include heterocyclic amines (HCA), fat, N-nitroso-compounds (NOC) and heme iron and are explained in more detail in section 1.4.3.

1.4.1 Red meat and colon cancer: epidemiology

The relationship between red meat consumption and colon cancer risk remains controversial. In 1997 the World Cancer Research Fund (WCRF, 1997) reviewed the epidemiological data and concluded that there is a probable association between the intake of red meat and colon cancer risk. It was suggested that if eaten at all, red meat intake should be limited to less than 80g per day and that it is preferable to choose fish, poultry or meat from non-domesticated animals in place of red meat. With the publication of additional epidemiological studies since 1997 there is now less evidence for an association between red meat intake and colon cancer risk (Truswell, 2002). One-third (n=30) of the case-control studies and 1/5 (n=15) of the cohort studies show a positive association between red meat intake and colorectal cancer risk (Truswell, 2002).

Variation in the results between epidemiological studies may be caused by other dietary or environment factors that are specific to the country in which the study was conducted (Hill, 2002). Cohort studies conducted in Europe do not show an association between red meat intake and colon cancer risk (Hill, 2002). Whereas a prospective study (Hsing et al, 1998) and two cohort studies from North America (Willett et al, 1990; Giovannucci et al, 1994) showed an increase in colorectal cancer risk for the highest intake group. These geographical differences in risk suggest that other lifestyle factors specific to these countries may influence the involvement of red meat in contributing to colon cancer risk.

1.4.2 Red meat and colon cancer: animal studies

The carefully regulated feeding of red meat to rats administered with a carcinogen to induce ACF or colon tumors has not provided a clear indication of the role red meat may play in promoting colon carcinogenesis. Studies that have compared red meat intake to other protein sources (mainly casein), have shown red meat to promote colon cancer risk in three studies (Goldin et al, 1980; Nutter et al, 1983; McIntosh et al, 1995) and to have no effect in five studies (Clinton et al, 1979; Nutter et al, 1983; Pence et al, 1995; Lai et al, 1997; Parnaud et al, 1998). A protective effect was only found for red meat when a mouse model was used which contradicts the rat data (Nutter et al, 1990). Further variation may have been caused by the use of different sources and quantities of red meat, the cooking method used and/or varying quantities of other dietary components such as fat that may interact with red meat. Epidemiological studies suggested that red meat may be more promotional than white meat however only one animal study has examined this relationship and found no effect of these meat sources on ACF expression (Parnaud et al, 1998). The lack of consistency in animal studies may be due to the other components added to the experimental diets. Most of these diets were AIN-76 based, with 5% fat, high calcium and high fibre. Using this as the background diet does not accurately portray the Western diet that contains high fat, low fibre and low calcium. Furthermore all of the studies used a moderate concentration of red meat, providing less than 24% protein in the diet. In the only study that compared the intake of moderate and high intake of red meat on ACF expression the difference in protein content of these two diets was not large (17% and 24% protein) (Parnaud et al, 1998). This may account for the lack of an effect of red meat on promoting ACF expression.

1.4.3 Red meat and colon cancer: current theories

The component(s) of red meat responsible for increased colon cancer risk are unclear. Current theories that are being actively investigated include HCA, fat, NOC and heme iron.

1.4.3.1 Heterocyclic amines

Heterocyclic amines (HCA) are a family of mutagenic and carcinogenic compounds produced by the pyrolysis of creatine, amino acids and monosaccharides which can occur during the cooking process of meat. The quantity of HCA that forms depends upon the type of meat and on the cooking technique (boiling, frying, barbequing, time and temperature) (Skog et al, 1995). HCA are true mutagens as they can cause DNA-mutations in-vitro (Pfau et al, 1999) and in animal models HCA induce tumors at various sites including the colon (Takayama et al, 1984; Ito et al, 1991). Furthermore, the addition of PhIP (the most abundant HCA generated in cooked meat) to a rodent diet has been shown to induce APC gene mutations (Burnouf et al, 2001). Therefore, it is possible that HCA may be involved in the initiation process of carcinogenesis as APC gene mutations occur in 85% of all sporadic and hereditary colorectal tumors in humans (Ransohoff and Lang, 1991).

Although HCA have induced tumors in rodent bioassays, the dosages required were much higher than estimates of human exposure levels (Augustsson et al, 1999). However, a recent study has shown that genotoxins can be released by the proteolysis of cooked beef, significantly increasing the amount of DNA-damaging material that was not extractible

prior to enzymic digestion. These findings suggest that human exposures to diet-derived genotoxins may have been underestimated (Martin et al, 2002).

Schiffman and Felton (Schiffman and Felton, 1990) postulated that the degree of done-ness of red meat maybe an important indicator of HCA intake. Two studies have shown that the done-ness of meat is related to increased colon and rectal cancer risk (Schiffman and Felton, 1990; Gerhardsson de Verdier et al, 1991) and one study has shown that there is no association (Muscat and Wynder, 1994). Variations in findings regarding epidemiological evidence may be due to the large variation in individual human susceptibility. Variations which include dosimetry, metabolism, carcinogenic potency and epidemiology are suggested to result in a 200 fold difference in risk between individuals for exposure to HCA (Felton et al, 1997). Further differences in the ability of the individual to repair DNA could cause a further ten fold variation (Felton et al, 1997). Thus, the risk of a particular individual suffering from colon cancer as a result of HCA exposure may be a thousand times greater or a thousand times less than the risk for another individual, depending on exposure and genetic differences (Felton et al, 1997).

1.4.3.2 Fat

Over the past two decades, the consumption of fat, particularly animal fat, has been implicated with increasing the risk of colon cancer (Giovannucci and Goldin, 1997). Animal studies have provided insight into various ways in which dietary fat may promote colon carcinogenesis. The most cited hypothesis is that dietary fat induces the excretion of bile acids which may be converted to secondary bile acids by colonic bacteria (Reddy,

1992). These secondary bile acids act as tumor promoters by a non-specific irritant effect that increases cell proliferation in the colonic mucosa (Narisawa et al, 1974; Lapre and Van der Meer, 1992). Another possible role of red meat fat promoting colon carcinogenesis is by the mitogenic effect of specific fatty acids. Diacylglycerides resulting from the incomplete digestion of dietary triglycerides may selectively induce mitogenesis of adenoma and carcinoma cells (Friedman et al, 1989). Finally, red meat fat may promote colon carcinogenesis by increasing insulin concentration systemically and by increasing insulin resistance. Reducing the dietary ratio of polyunsaturated fat:saturated fat has been shown to improve insulin sensitivity. As beef fat has a much lower ratio of polyunsaturated fat:saturated fat as compared to other commonly used meat fats such as chicken, diets high in beef fat may increase insulin resistance and colon cancer risk (Linscheer and Vergroesen, 1988).

1.4.3.3 N-nitrosocompounds

N-nitrosocompounds (NOC) can be produced endogenously in the colon by a two step process. Amino acids are converted to amines and amides by bacteria in the colon, which then undergo N-nitrosation to form NOC (Mirvish, 1995). The dietary source of amino acids is important for the formation of NOC as red meat, but not white meat, influences NOC concentration in the feces (Bingham et al, 1996; Bingham et al, 2002). Red meat concentration in the diet is directly related to the concentration of NOC in the feces (Hughes et al, 2001; Bingham et al, 2002). At the higher levels of meat consumption concentrations of NOC were found to be of the same order of magnitude as the concentration of tobacco-specific NOC in cigarette smoke (Ohshima and Bartsch, 1981).

At present, however, there is not any direct evidence that these NOC agents cause genotoxicity or carcinogenicity in the colon. There are many different types of NOC, some of which are alkylating agents known to induce GC to AT transitions at the second base of codon 12 or 13 of the K-ras gene (Bos, 1989). This mutation is common in colorectal cancer cases, occurring in 50% of colon adenocarcinomas (Bos, 1989) and may be a way in which a high red meat diet may initiate and/or promote colon carcinogenesis.

1.4.3.4 Heme iron

Babbs (1990) hypothesised that high amounts of unabsorbed fecal iron resulting from excessive dietary iron may catalyse the production of oxygen radicals, thus increasing fecal mutagenicity, activating carcinogens or forming tumor promoters within the intestinal lumen. However, there is mixed evidence for an association between high dietary iron intakes and colon cancer risk (Nelson, 1992). The variation in results may be due to the form of iron given. Sesink et al (1999) investigated the effect of different dietary iron sources on risk markers of colon cancer in a rat model. Heme iron, in comparison to ferric citrate, protoporphyrin IX and bilirubin, produced a highly cytotoxic environment in the colon as shown by an increase in cell proliferation, lipid peroxidation and fecal water cytotoxicity. These findings are supported by carcinogen induced cancer studies in rats that showed heme iron intake to increase ACF size and number and increase tumor expression (Sawa et al, 1998; Pierre et al, 2003). Alternatively, studies that have fed high concentrations of non-heme iron to rats administered with a carcinogen have shown little to no effect on ACF expression (Davis and Feng, 1999; Wright et al, 1999).

The difference in colon cancer risk between heme and non-heme iron may be due to the different ways in which these molecules oxidise lipid. When heme iron compounds (myoglobin, hemoglobin, cytochrome c, hemin and hematin) react with the lipid hydroperoxide (ROOH), the lipid alkyl peroxyl radical (ROO) is predominately generated. This radical species has been shown to exert strong cytotoxic effects on bacterial cells (Akaike et al, 1992; Akaike et al, 1995), and can cause whole cell effects that include the leakage of potassium ions and impairment of enzyme activity (Van der Zee et al, 1989). The genotoxic effects of this radical species have also been demonstrated in vitro. ROO has been shown to cleave plasmid DNA (Sawa et al, 1998) and produce abasic sites in calf thymus DNA (Kanazawa et al, 2000). Furthermore, the ROO radical has a long half life of more than 30 minutes which potentially allows access to distant sites, whereas the short lived radicals, such as the hydroxl radical may not (Peak and Peak, 1990; Akaike et al, 1992). Non-heme iron oxidises differently to heme iron by generating the alkyl radical (R) and/or the RO radical. When these radicals were exposed to DNA at the same concentration as the ROO radical, DNA damage was not induced (Akaike et al, 1992; Sawa et al, 1998).

The type of dietary fat may be an important factor affecting the production of radical species from iron in the colon. Both animal and in vitro studies have shown that polyunsaturated fats increase colon cancer risk in comparison to saturated fats (Sawa et al, 1998). The presence of two or more double bonds within polyunsaturated fatty acids increases their susceptibility to oxidation in comparison to saturated fatty acids containing no double bonds. A study by Chin and Carpenter (1997) investigated the

interaction between non-heme iron (ferrous fumerate) and fat type (corn oil, menhaden oil and beef tallow) and its effect on risk markers for colon cancer. Although colonocyte proliferation was unaffected by diet, comparing the high iron diets, rats fed corn oil had significantly higher peroxidised lipids in the feces compared to the beef fed rats. A trend was also shown for increased peroxidised lipid in the mucosa of the rats fed high iron and corn oil compared to the high iron and beef fed rats. This suggests that dietary iron is peroxidising polyunsaturated fat in the colon and may also be damaging the mucosa. Further effects on lipid peroxidation and proliferation may have been seen if heme iron had been used as the iron source, as used in the study by Sesink et al (1999).

1.5 Whey protein

Whey is the yellow-green liquid that separates from the curd during manufacture of cheese and casein. Whey contains a high proportion of water (approximately 87%) with only 0.7% protein. Protein can be isolated from whey by ultra-filtration, evaporation and spray-drying at temperatures between 150 and 200°C (Caric, 1994). The resulting protein product is a dry powder that contains approximately 80% protein. By changing the drying temperature and by modifying the processing protocol, it is possible to produce whey protein products that vary in their composition, eg altered amino acid composition, varying quantities of protein, fat, lactose and minerals (Smithers et al, 1996). The non-protein components of whey protein concentrate can be removed by ion exchange or microfiltration. This step removes a majority of the fat, lactose and denatured protein resulting in a product that contains up to 94% protein (whey protein isolate) (**Table 1.2**).

Table 1.2. Composition of whey protein concentrate and whey protein isolate

	Whey protein concentrate	Whey protein isolate
Protein content	78-88%	90-94%
Protein denaturation	3-10%	< 2%
Fat content	4-7%	< 1%
Lactose content	2-10%	< 1%

(New Zealand Milk Products, www.nzmp.com/)

Whey protein is primarily composed of β-lactoglobulin and α-lactalbumin, two small globular proteins that account for approximately 70-80% of total protein in the whey protein product. Minor protein components include glycomacropeptide, bovine serum albumin, lactoferrin, immunoglobulins, phosopholipo-proteins and numerous bioactive enzymes and factors (IGF-I and II, platelet derived growth factor, IGF-BP, acidic and basic fibroblast growth factors) (Smithers et al, 1996).

The main dietary source of whey protein is cows' milk which contains approximately 0.8g/100ml and ricotta cheese, containing 10-11g/100g whey protein. Most hard cheeses do not contain any whey protein as it is removed during manufacturing. At an increasing rate, whey protein is being added to processed foods like pasta. Whey protein is chosen as it has very good manufacturing properties such as gelling strength, antioxidant properties and its tastelessness which means that its addition does not influence the flavor of the final product.

1.5.1 Whey protein and colon cancer: epidemiology

The study of colon carcinogenesis in humans is quite difficult, with ethical and legalistic considerations often limiting the scope of experimentation. It is even more difficult when dietary intervention studies are proposed which examine products that are not commonly consumed in large quantities. Consequently, there have been very few studies examining potential anti-cancer effects of whey proteins in humans. The two studies that have been conducted have examined the effect of whey protein on urogenital, breast, pancreas and liver cancers (Kennedy et al, 1995; Bounous, 2000). A phase I-II clinical study of five patients with metastatic cancer of the breast, one of the pancreas and one of the liver were fed 30g of whey protein for six months (Kennedy et al, 1995). Whey protein along with chemotherapy was shown to be effective in aiding tumor regression in two patients, to stabilize tumor growth in another two patients and have no effect in the remaining three patients. Bounous (2000) has also presented various case reports that suggest that dietary supplementation of whey protein has anti-tumor effects in urogenital cancer. Although both these studies do suggest possible anticancer effects of whey protein large case controlled studies are required to strengthen these findings.

1.5.2 Whey protein and colon cancer: animal studies

Bounous et al (1988) was the first to demonstrate the protective effects of whey protein against dimethylhydrazine (DMH) induced colon cancer. These findings are supported by a study by Papenburg et al (1990) that showed diets containing whey protein reduced the number and size of DMH induced colon carcinomas by four fold in comparison to diets containing casein and Purina lab chow. Hakkak et al (2001) showed a significant

reduction in tumor incidence for rats consuming a WPI diet in comparison to a casein diet during lifetime exposure in the first generation (30% vs 56% incidence) and second generation of offspring (29% vs 50% incidence). In these studies the effect of whey protein was studied in relation to an AIN-93 diet with low fat. In a high fat Western diet whey protein and casein had a similar effect on colon tumor expression which was significantly less than red meat and soy protein (McIntosh et al, 1995).

1.5.3 Whey protein and colon cancer: current theories

The effect of α-lactalbumin (a component of WPC) on cancer cell growth *in vitro* has been examined. Colon cancer cells (HT-29 and Caco-2) grown in media containing α-lactalbumin initially increased cell proliferation. However, this increase in growth was subsequently followed by cessation in proliferation and a drop in viability (Sternhagen and Allen, 2001). The addition of a multimeric form of α-lactalbumin promoted apoptosis in colon cancer cells, while sparing various types of mature epithelial cells (Hakansson et al, 1995). These effects in cell growth were not seen when a human hepatoma cell line (Hep G2) was exposed to whey protein product rich in α-lactalbumin (Tsai et al, 2000). This suggests that the colon cancer cells may be more responsive to the cytotxic or apoptotic effects of α-lactalbumin than liver cancer cells. However, differences in effect may also be due to the different forms of α-lactalbumin used in the studies.

Findings from animal studies have led to a number of hypotheses as to how whey protein may be acting to inhibit the carcinogenic process. These systems include stimulation of cellular glutathione concentration, stimulation of phase I and phase II enzymes by

lactoferrin and changes in fat and bile acid metabolism. Ways in which these systems may influence carcinogenesis are outlined below.

1.5.3.1 Glutathione stimulating

The tri-peptide glutathione (GSH) plays an important role in enzyme activity, metabolic and cell cycle related functions in virtually all cells (Meister and Anderson, 1983). Its ability to directly scavenge free radicals and act as a co-substrate in the glutathione peroxidase catalysed reduction of hydrogen peroxide and lipid hydroperoxides makes GSH central to defense mechanisms against intra- and extra-cellular oxidative stress (Cotgreave and Gerdes, 1998). In addition, GSH and glutathione transferase are major components involved in the metabolism of xenobiotics (Dekant, 1996).

A primary way in which GSH can be increased is by increasing the delivery of cysteine to a cell as this is the rate limiting amino acid in GSH synthesis. This is difficult since cysteine can be toxic, it is not transported efficiently into cells and is oxidized spontaneously at neutral pH (Nishiuch et al, 1976; Meister et al, 1986). Whey protein may be an effective way to supply cells with cysteine as it is one of the richest protein sources of this amino acid. In patients where glutathione concentration is depressed (HIV and obstructive airway disease), dietary supplementation of whey protein has been shown to raise plasma glutathione concentration to normal (Lothian et al, 2000; Micke et al, 2001). In an animal study, whey protein in comparison to other protein sources low in cysteine was shown to elevate hepatic glutathione concentration but did not effect plasma or colon mucosa glutathione concentration (McIntosh et al, 1995). However, a study by

Hakkak et al (2001) did not show an effect of whey protein on hepatic GSH levels in rats in comparison to casein. This lack of an effect may have been due to the addition of essential amino acids to the diet to balance for amino acids supplied by the proteins. Whey protein has also been shown to increase the activity of GST-α activities in the liver of female rats administered with dimethylbenz(a)anthracene (Rowlands et al, 2000). This suggests that whey protein can increase GST enzyme activity.

1.5.3.2 Lactoferrin stimulation of phase I and II enzymes

The anticarcinogenic effect of whey protein may be due to the presence of lactoferrin. Whey protein contains 0.4-1.3g of lactoferrin/100g (based on figures from Smithers et al (1996)). Therefore in a diet containing 20% whey protein, there will be 0.08-0.26g of lactoferrin per 100g diet. Animal studies have shown that a minimum of 0.2% lactoferrin in the diet is sufficient to exert protective effects against the expression of AOM-induced tumors in the rat (Sekine et al, 1997; Tsuda et al, 2000). Lactoferrin may inhibit carcinogenesis by suppression of phase I enzymes, such as cytochrome P450 1A2 (CYP1A2), which is preferentially induced by carcinogenic heterocyclic amines (Tsuda et al, 1999). It may also enhance phase II enzyme activity or alter immune system status. Lactoferrin may also be involved with antimicrobial activity (Van der Strate et al, 2001).

1.5.3.3 Fat and Bile acid metabolism

A study by Kruidenier et al (1985) showed that whey protein has the ability to bind bile acids, particularly at low pH. Equilibrium dialysis studies on rat stomach were used to examine the effect of whey protein on bile acid induced sodium secretion and hydrogen

loss. In this acidic environment, whey protein protected mucosal membranes from the damaging effect of bile acids. Although whey protein was not tested in the rat intestine model, it was shown that bovine serum albumin was able to reduce the effect of bile acid on water and solute transport in the jejunum and inhibited deoxycholate absorption (Kruidenier et al, 1985). It is possible to speculate that if whey protein is able to bind bile acid in the digestive tract this may increase the amount of bile acid synthesised by the liver to ensure effective emulsification of fat in the intestine. Furthermore, an increase in the concentration of bile acids in the feces and a decrease in liver and/or plasma cholesterol would be seen.

Only a limited number of studies that have examined the hypocholesterolemic effects of whey protein, have measured the fecal excretion of bile acids. Whey protein was shown to slightly increase fecal bile acid excretion in rats fed 23% whey protein compared to casein (Sautier et al, 1983). This effect was further enhanced in a study using rabbits where whey protein, in comparison to casein significantly increased the excretion of total bile acids (Lovati et al, 1990). Paradoxically, Zhang and Beynen (1993) showed that in rats fed 30% whey protein there was a decrease in the excretion of fecal 3-α bile acids in comparison to rats fed 15% whey protein or casein at both concentrations. The cholesterol lowing ability of the 30% whey protein in the study was possibly due to lower very low density lipoprotein cholesterol which suggests a decreased synthesis of cholesterol in these rats.

As the main role of bile acids is to emulsify fat, the enhancement of bile acid secretion by whey protein may effect the digestion and utilization of fat. In a high fat diet, whey protein was shown to reduce fecal fat and the mesenteric fat mass of animals in comparison to those fed red meat as the protein source (McIntosh et al, 1995). This suggests that there may be an increased uptake and utilization of dietary fat as a result of whey protein. Unfortunately fecal bile acids were not measured in this study.

1.6 Concentration of dairy protein: colon cancer risk

The degree to which dairy protein concentration influences risk markers of colon cancer has been studied in only a few animal studies and the results are not clear. A study by Tatsuta et al (1992) investigated the effect of dietary protein concentration on the expression of AOM-induced colon tumors. They showed that the administration of low (10%) and very low (5%) protein diets (as casein) compared to normal protein diet (25%) significantly increased the incidence and number of colon tumors. This increase in colon carcinogenesis was related to the increase in nor-epinephrine level and cell proliferation in the colon wall. However, a study by Clinton et al (1992) showed no significant influence of protein concentration (as casein) on intestinal tumors and concluded that energy intake and fat was the influential factor on tumor expression. These two studies only examined the effect of the concentration of casein on colon cancer risk. The effect of whey protein on colon cancer risk has not been examined.

1.7 Whey protein: Body weight effects

There is increasing evidence that whey protein may reduce weight gain of rats in comparison to other sources of animal protein in the diet (**Table 1.3**). In comparison to casein, four studies have shown that whey protein intake reduced weight gain (Hakkak et al, 2000; Minehira et al, 2000; Badger et al, 2001; Jacobucci et al, 2001), whereas five studies showed no effect (Sautier et al, 1983; Bounous et al, 1988; Papenburg et al, 1990; Zhang and Beynen, 1993; McIntosh et al, 1995). In the four studies that reported a reduction in weight gain, only one study reported a reduction in diet intake (Jacobucci et al, 2001) whereas the other three studies either did not measure diet intake (Hakkak et al, 2000; Badger et al, 2001) or showed no change (Minehira et al, 2000). Therefore, it is not clear whether whey protein can reduce body weight gain independent of energy intake. The studies summarized in **Table 1.3** primarily examined the effect of dietary protein on weight gain in young growing rats. Minehira et al (2000) also studied the effect of whey protein and casein on weight gain in mature rats (8 month old). In that study significant reductions were seen in body weight gain in both young and old rats (**Table 1.3**). This suggests that whey protein may be effective in reducing body weight gain in mature rats when weight gain is primarily due to fat deposition rather than muscle and bone growth.

In comparison to red meat fed rats, whey protein intake reduced final body weight of rats by 10% (McIntosh et al, 1995). A 30% reduction was also seen in mesenteric fat storage which suggests that the reduction in weight gain for whey protein fed animals may be due to altered fat metabolism, utilization and/or storage.

A possible mechanism whereby whey protein may affect weight gain and fat utilization and/or storage is by increasing the circulating concentration of plasma tryptophan. Whey protein is the richest protein source of tryptophan containing between 2 and 2.9 g/100g depending upon the isolation method used in processing (NZ milk products, www.nzmp.com/). Other dietary protein sources such as casein (1.16 g tryptophan/100g) (Zhang and Baynen 1993) and lean beef (1.2 g/100g of tryptophan) contain considerably less tryptophan. Human studies have shown that supplementing the diet with tryptophan can increase plasma cortisol and ACTH (Modlinger et al, 1979; Modlinger et al, 1980). However, the quantity of dietary trypophan required to produce a similar response in plasma cortisol in the rat is not known. The primary effect of cortisol on metabolism is to increase the concentration of plasma glucose at the expense of proteins and fat stores. This is done by stimulating hepatic gluconeogenesis (conversion of amino acids to glucose), proteolysis (degradation of tissue protein) and lipolysis in adipose tissue to release free fatty acids (Sherwood, 1997). Therefore, whey protein intake may stimulate plasma cortisol concentration by increasing the circulating concentration of tryptophan. The adverse effect of cortisol induced proteolysis may be prevented in a high protein diet by the large supply of dietary amino acids, which are utilized prior to muscle stores.

Table 1.3. Summary of body weight and diet intake data from whey protein rat feeding studies.

Author	Diet specifications	Time diet fed (days)	Age/Weight rat (start of study)	Body weight (BW) or Wt gain (WG) (g)	Diet intake (g/day)
Badger et al, 2001	AIN-93	55	birth	Cas = 278 (m) } *	Not reported
	5% corn oil			WPI = 215 (m)	
	amino acid balanced			Cas = 181 (f)	
				WPI = 167 (f)	
Hakkak et al, 2000	AIN-93G	180	birth	F1 casein = 300 } *	Not reported
	5% corn oil			F1 WPC = 260	
	amino acid balanced			F2 casein = 290 } *	
				F2 WPC = 250	
Jacobucci et al, 2001	20% protein	45	30-35g	Cas = 199.9 } *	21.6 } *
				WPC = 159.2	18.3
Marquez-Ruiz et al, 1992	22% fat (2% corn oil + 20% butter)	42	133g	Cas = 378.5	Not reported
	22% protein			WPC = 350.7	
McIntosh et al, 1995	20% fat (SSO/lard)	150-180	5wk	Cas = 792	19.4
	0.5% calcium			WPC = 780	19
				Meat = 871	20.2

McIntosh et al , 1998	AIN-93	150	6 month old	WPC = 943	Not reported
				BBQ beef = 1010	
Minehira et al, 2000	AIN-93	21	4 wk old	Cas = 151 } *	18
	10% soy bean oil			WPI = 120	17.5
	20% protein		8 month old	Cas = 60 } *	21.6
				WPI = 35	21.5
Nagaoka et al, 1991	2% corn oil	68	90g	Cas = 167	15.1
	25% protein			WPC = 162	14.8
Sautier et al, 1983	8% Maize oil	49	140g	Cas = 432	347.4 #
	23% protein			WPC = 420	325.6 #
Zhang & Beynen, 1993	3% soybean oil	21	28 days	Cas 15% = 176	13.9 } *
	9% coconut fat			Cas 30% = 187	13.2
				WPC 15% = 174	12.1 }
				WPC 30% = 174	11.9

* denotes denotes significant difference between groups, $p<0.05$. # denotes different units, values are expressed in kJ/day.

WPC, whey protein concentrate, WPI, whey protein isolate, Cas, casein. Generation 1 (F1) and generation 2 (F2)

1.8 Specific Aims and objectives of this thesis

The aim of this research was to gain insight into how dairy and red meat influences metabolism and colon cancer risk. To investigate this aim, animal studies were designed to determine:

1. the effect of fat type and heme iron on risk markers of colon cancer.
2. whether a PUFA rich diet containing heme iron can induce genotoxicity in colonocytes of rats.
3. whether a diet high in red meat increases colon cancer risk by using ACF expression as a biomarker of risk.
4. whether a diet high in whey protein reduces colon cancer risk by using ACF expression as a biomarker of risk.
5. whether increasing the density of whey protein in the diet reduces body weight and fat storage and improves insulin sensitivity in comparison to red meat.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Animals

Outbred Wistar rats ten to thirteen weeks of age, were obtained from the Laboratory Animal Services Branch, Adelaide University. At this age the animals have finished the main growing stage and are young adult rats. Animals were housed in wire cages to prevent the consumption of bedding material and coprophagy. They were maintained in an air-conditioned environment of 23 ± 2 °C with a 12-h light/12-h dark cycle. They were given free access to the diet and deionised water. Rats were weighed weekly. All experimental procedures using animals were approved by the Commonwealth Scientific and Industrial Research Organisation, Health Sciences and Nutrition Animal Ethics Committee and the University of Adelaide Animal Ethics Committee.

2.1.1 ACF induction protocol

After four weeks on the experimental diets rats were injected subcutaneously once a week for two weeks with AOM (Sigma, St Louis, MO) dissolved in normal saline at a dose of 15 mg/kg body weight.

2.2 Experimental Diets

All diets were based on a modified AIN-93 semi-purified diet to simulate the Western diet (**Table 2.1**). The amount of fat in the diet was modified to a level of 20% and the type of fat varied between experiments. The fibre was supplied as α-cellulose at 2%. The calcium was supplied as calcium carbonate at 0.1%. Iron was balanced between dietary treatments using ferric citrate. The quantity of Vitamin E provided by sunflower seed oil was balanced between diets to the AIN-93 recommended amount.

Table 2.1. The composition of standard and modified AIN-93 diets

Ingredient	AIN-93G diet[1]	Modified AIN-93 diet
Casein	20.0	20.0
Fibre	5.0 (as solko-floc)	2.0 (as α -cellulose)
Cornstarch	39.7	33
Dextrinized cornstarch	13.2	-
Sucrose	10.0	20
Fat	7.0 (soy bean oil)	20 (various)
Vitamin mix (AIN-93M-VX)	1.0	1
Mineral mix (AIN-93M-MX)	3.5	3.5
L-Cysteine	0.3	0.3
Choline	0.25	0.2
Tert-butylhydroquinone	0.0014	-

All values in g/100g

[1]As outlined in Reeves et al (Reeves et al, 1993)

2.2.1 Dietary protein and amino acid requirements for growth and maintenance

The different amino acid requirements for the growth of young rats and the maintenance of adult rats are shown in **Figure 2.1**. Young rats require 5 to 10 fold more amino acids than adult rats. Cysteine and methionine are particularly important for growth as a diet deficient in these amino acids in comparison to the other amino acids has the largest effect on nitrogen balance (Yoshida, 1990).

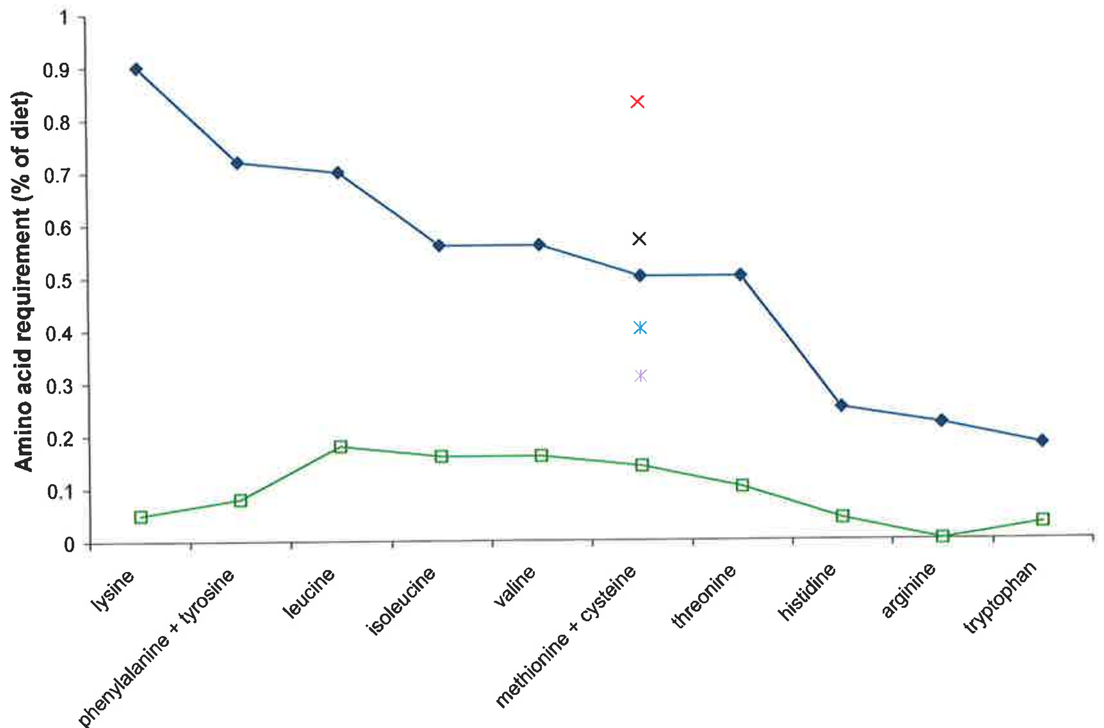


Figure 2.1 Requirement of essential amino acids for maxiamal growth of young rats (◆) (Rama Rao et al, 1961) and for nitrogen equilibrium in adult rats (□) (Ashida and Yoshida, 1975). The amount of cysteine and methione provided by the following diets is shown; AIN-93G (x), AIN-93M (x), 8% red meat (*) and 8% whey protein (*).

There are two main AIN-93 diets that accommodate the different needs of a young (AIN-93 growth) and adult rat (AIN-93 mature) (Reeves et al, 1993). The AIN-93 growth diet contains 17g protein/kg diet (as casein) and 3g cysteine/kg of diet. Therefore, the total cysteine and methionine content of the diet is approximately 8.3 g/kg of diet which is more than sufficient for proper animal growth (**Figure 2.1**). As adult rats do not require as much essential amino acids and protein as growing rats, the AIN-93 maintenance diet only contains 12g protein/kg diet (provided by casein) and 1.8g cysteine/kg diet. Adult rats on this diet consume 5.7g of cysteine and methione/kg of diet which is more than double the required amount (**Figure 2.1**).

In Chapters 5 and 6 of this thesis, red meat and whey protein was added to diets to provide 8, 16 or 32% protein. As adult rats were used in these studies, the two dietary protein sources provided sufficient quantities of essential amino acids (cysteine and methionine) for growth maintenance even in the 8% protein diets. Therefore additional amino acids were not added. The composition of red meat and whey protein concentrate is outlined in **Table 2.2**, and includes fat, mineral and amino acid composition.

Based on AIN-93 recommendations, a moderate protein diet has been characterized as one that contains between 12 and 20% protein. Diets containing 8% protein or less have been characterized as low protein diets as they only provide the minimum amount of essential amino acids for maintenance. Diets containing protein levels above 25% are termed high protein diets.

Table 2.2 The fat, mineral and amino acid composition of red meat and whey protein

	Red meat	Whey protein
Fat		
Total (g/100g)	15	6
Saturated (%)	45.8	62.4
Monounsaturated (%)	46.3	31.8
Polyunsaturated (%)	7.9	5.8
Minerals (ug/ml)		
Iron	180	4
Sodium	2000	10,400
Potassium	10,400	1,500
Calcium	140	1,700
Phosphorus	6,200	8,500
Protein		
Total (%)	79.3	79
Cysteine	1.9	2.9
Methionine	2	2.1
Aspartic acid	7	11
Threonine	2.3	5.7
Serine	2.8	5
Glutamic acid	13	17.7
Glycine	4.6	2.4
Alanine	5.1	5
Valine	3.8	5.7
Isoleucine	3.6	4.5
Leucine	6.5	12.6
Tyrosine	2.8	3.8
Phenyalanine	3.4	3.8
Histidine	2.2	2.3
Lysine	7	10
Arginine	5.4	3
Proline	3.8	5.2

2.2.2 Induction of insulin resistance with high fat (30%) diet

The animal model of insulin resistance used in this thesis was the high fat (30%w/w) fed Wistar rat. First identified by Storlien (1986), animals fed a high fat diet for 3 to 4 weeks developed insulin resistance. Insulin resistance was identified as an increase in fasted plasma insulin concentration, a reduction in glucose uptake by skeletal muscle and adipose tissue, a failure to suppress liver glucose output by insulin and an accumulation of muscle triglyceride (Kraegen et al, 1986; Storlien et al, 1986; Kraegen et al, 1991; Storlien et al, 1991; Oakes et al, 1997). In the long term, feeding of the high polyunsaturated fat (30% w/w) diet for 10 months, further increased fasting hyperinsulinemia and increased body weight (30%) and body fatness (60%), in comparison to chow-fed animals (Chalkley et al, 2002). These metabolic changes are similar to those developed in the genetically predisposed model of obesity and insulin resistance, the Zucker fatty rat (Bray, 1977; Storlien et al, 1986; Chalkley et al, 2002). However, the Zucker fatty rat also develops hypertriglyceridemia which does not occur in the high fat diet fed Wistar rat. In spite of this difference, a study by de Souza et al (2001) showed that a pharmacological drug (Nateglinide) was equally effective in improving glycaemic control in a high fat diet fed Sprague-Dawley rat as a Zucker fatty rat (fa/fa).

The type of fat used in this animal model of insulin resistance may influence the degree to which insulin resistance develops (Storlien et al, 1991). Using the high fat (30%) diet saturated fat significantly increased insulin resistance, long- and short-chain omega(3) fatty acids significantly reduced it, whereas the effects of monounsaturated and omega(6) polyunsaturated fatty acids ranged somewhere in between the two.

2.3 Colon epithelial biomarkers

2.3.1 ACF

Following removal from the rat, the colon was assessed for ACF following the method of McLellan and Bird (1988). The colon (cecum to anus) was removed and rinsed with saline, opened longitudinally, flattened onto blotting paper and fixed in 10% buffered formalin overnight. The tissue was divided into two equal segments (proximal and distal) between the distal Peyers patch and the Herring bone musculature and stained with 0.2% methylene blue in phosphate-buffered saline for 20 min. Aberrant crypts were distinguished from the surrounding normal crypts if they fulfilled the following criteria: increased size, slit like lumen, thicker cell wall and were raised above the crypt surface (Bird, 1987). Using a light microscope (40 x magnification), the number of aberrant crypts and foci were recorded in the proximal and distal colon.

2.3.2 Genotoxic damage in colonocytes

2.3.2.1 Colonocyte isolation

Various techniques have been used to isolate colonocytes from the colon (Pool-Zobel et al, 1993; Brendler-Schwaab et al, 1994; Anderson et al, 1999). These techniques are compared in Chapter 4 of this thesis. The optimal method is outlined below.

A 5cm section of rat colon is rinsed with pre-warmed HBSS 2 to 3 times for a total of 10 min. The colon is then filled with 40 mM dithiothreitol (DTT) in HBSS at room temperature for 10 min to dispel the mucus. Following a brief rinse of the colon with HBSS, the colon is then filled with 2 to 3 mls of digestive buffer (1.22mg proteinase K/ml HBSS) and then sutured. The colon is then placed in a 50 ml Falcon tube that has

been half filled with HBSS and incubated whilst on a platform shaker for 30 min. The colon is then removed and the digestive buffer containing the colonocytes is removed. The suspension is centrifuged for 8 min at 100g. After removal of the supernatant the cell pellet is resuspended in 5-10 mls of Rosewell Park Memorial Institute Medium (RPMI) 1640.

Cell membrane integrity, which correlates closely with cell viability, was determined by trypan blue exclusion. A sub-sample of approximately 150,000 colonocytes were placed in an ependorf. They were then centrifuged at 100g for 5 min and the supernatant was removed. The cell pellet was resuspended by triturating with 50 μl of 0.2% trypan blue (Sigma Chemicals, Missouri, USA) and 50 μl of PBS. After 5 min the Trypan blue-cell suspension was added to a hemacytometer. Using a light microscope with 200 x magnification, the viable (unstained) and non-viable cells (stained dark blue) were counted. Cell viability was calculated by dividing the number of viable cells by the total number of cells. This number was multiplied by 100 to express the values as a percentage.

2.3.2.2 Comet assay

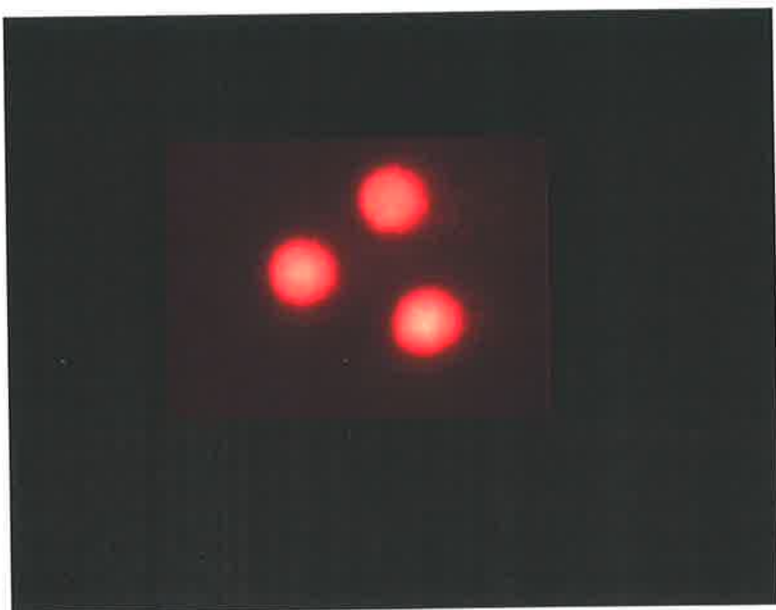
The Comet assay was performed according to McKelvey-Martin et al (McKelvey-Martin et al, 1993) and Pool-Zobel et al (Pool-Zobel et al, 1997). Microscope slides were covered with 1% normal melting agarose (Sigma Chemicals, Missouri, USA) in PBS and then placed in an oven at 50°C. Once the agarose had dried, 85 μl of normal agarose (0.5% in PBS) was applied on top of the first layer along with a coverslip. The slide was then placed in a humidified box at 4°C for at least 10 min to allow the agarose to set. Cell culture media was removed from the colonocytes by centrifugation at 100 g followed by

aspiration of the media. The cell pellet was resuspended in 75 µl of low melting agarose (0.75% in PBS) and then added to the microscope slide. The agarose was allowed to solidify at 4°C before a final layer of low melting agarose (75 µl, 0.75% in PBS) was added.

All slides were placed in a lysis solution for atleast 1 h at 4°C (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium N-laurylsarcosine, 1% Triton X-100 and 10% dimethylsulphoxide, pH 8.0) Following lysis the slides were transferred to an electrophoresis tank (Maxicell® Primo, E-C Apparatus Corporation, New York, USA) containing ice-cold electrophoresis buffer (1 mM Na2EDTA and 300 mM NaOH, pH 13.5) for 20 min to allow the DNA to unwind before applying the current of 300 mA at 25 V for 20 min. After electrophoresis, slides were rinsed with neutralizing buffer (0.4 M Tris, pH 7.5) and stained with propidium iodide (30 µl in 1 mL 2 x standard saline citrate.

Images of DNA on the slides were captured at 200 X magnification using an Olympus BX41 microscope (Olympus, Victoria, Australia) with an Olympus Reflected Fluorescence System (Olympus, Victoria, Australia). A Spot digital camera with Spot RT software (Spot Diagnostic Instruments Inc., Michigan, USA) was used to digitally capture the images (**Figure 2.2**). The tail length and intensity (percentage of fluorescence in the Comet tail) of 50 randomly selected cells on each slide were analysed using Scion image analysis software available at http://rsb.info.nih.gov/nih-image/ (Helma and Uhl, 2000).

a.



b.

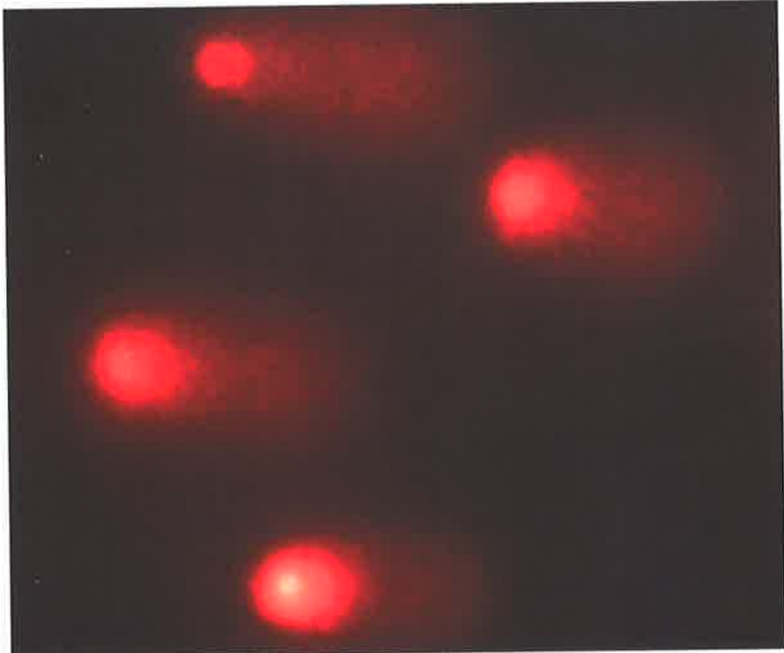


Figure 2.2 Images from the Comet assay of colonocytes treated with either a) PBS (negative control) or b) hydrogen peroxide (positive control). DNA of cells was stained with propidium iodide and the images are magnified 200 X.

2.3.3 Proliferation (PCNA)

Sections of distal colon were processed, mounted in wax blocks and 4 µm sections were cut by using a Leitz microtome. Slides were dewaxed, rehydrated and then incubated in 3% hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity. Sections were then hydrolysed in 2N HCl for 30 min and incubated in 10% normal horse serum (Vector, California, USA) for a further 30 min. Monoclonal murine anti-PCNA antibody PC-10 antibody diluted 1/1000 (Neomarkers, California, USA) was then applied to sections and incubated for 90 min. A negative control, tris buffered saline (TBS) was applied to one section instead of the anti-PCNA antibody. Following a rinse in TBS, the sections were incubated with biotinylated antimouse IgG (Vector, California, USA) for 30 min. Sections were then rinsed in TBS and incubated with conjugated streptavidin peroxidase diluted 1/1000 (Rockland, Philadelphia, USA), for a further 30 min before being stained with 0.25% 3, 3-diaminobenzidine (Sigma Chemicals, Missouri, USA). Sections were counterstained with Mayers Haematoxylin for 20 seconds prior to dehyradation and coverslipping. Slides were scored by examining 20 complete half crypts. The number of stained and unstained cells in the crypt was recorded. Proliferating cells were reported as a percentage of total cells in the crypt.

2.4 Fecal biomarkers

2.4.1 Fecal and cecal water

Fecal water was prepared following the method of Lapre et al (1991) and Sesink et al (1999). Approximately two grams of freeze dried feces was reconstituted with milliQ water to 30% of dry weight. Cecal contents were diluted with isotonic saline (0.5g/3ml) to obtain sufficient sample (Coleman et al, 2002). The samples were vortexed and then mixed in an incubator at 37°C for 1 h and centrifuged at 26,000 g for 30 min. The supernatant was removed and the osmolarity checked. If the osmolarity differed from 300 mOsM further water was added to another sample of freeze dried feces to obtain an osmolarity of 300 mOsM. Fecal water was stored at -20°C until analysis.

2.4.2 Culturing of HT-29 cells

The human colon cell line HT-29 was cultured in monolayers at 37°C with 5% atmospheric CO2 until cells were semi confluent. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Trace Bio-Science, Victoria, Australia) that contained 10% fetal calf serum, 20 mM glutamine and 1% penicillin/streptomycin. For use in the cytotoxicity assay or genotoxicity assay, cells were detached after incubation with 0.25% trypsin-EDTA at 37°C, and resuspended in DMEM.

2.4.3 Fecal and cecal water cytotoxicity

The cytotoxic effect of cecal and fecal water was quantified using the human adenocarcinoma HT-29 cell following a modified method of van Munster et al (1993). In brief, the HT-29 cells were plated in 96 well plates (15,000 cells/well). Cells were incubated for 24 h to give time for them to adhere to the plate. Media was then removed

and the cells were exposed to sterile filtered (Millipore filter, 0.5μm) fecal or cecal water for 1 h. The water extracts were then removed and the cells were rinsed with PBS. Fresh DMEM was then added to the cells and they were incubated for a further 48 h. Cell survival was determined by adding 25 μl MTT (tetrazolium salt 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide, Sigma Chemicals, USA) to the cells. Cell survival was read at an absorbance of 570nm using a SpectraMAX 250 ELISA Plate Reader (Molecular Devices, California, USA) and was expressed as a percentage of maximal absorption of control cells incubated with PBS.

To compare the cytotoxicity of cecal and fecal water samples from different dietary treatment groups, the concentration of cecal or fecal water that killed 50% of the cells was calculated (IC-50 value) (Kirana et al, 2003). To obtain the IC-50 value, cecal and fecal water samples were diluted by 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 using 0.9% saline. Each dilution was assessed using the cytotoxicity assay and the percentage of cells killed was plotted on a graph. The dilution at which 50% of the cells were killed was reported.

2.4.4 Heme iron

The amount of intact heme in the fecal water fraction of feces was measured by the Hemoquant assay (Schwartz et al, 1983) using hemin as the standard. This assay involves two separate incubations of each fecal water sample. One sub-sample is incubated with an oxalic acid solution (2.5 M oxalic acid, 90 mM ferrous sulphate ($FeSO4.7H2O$), 8.4 mg uric acid/L and 9.1 g mannitol/L) which converts the remaining fecal heme to fluorescent porphyrin. The second sub-sample is incubated with a citric acid solution (1.5

M citric acid) which extracts porphyrin already present in the fecal water. Following incubation at 100°C for 30 min, the porphyrins were purified by a three step process. In the first step, 250μL of the supernate from the oxalic acid or citric acid solution was mixed with 1.5 mL of a mixture of ethyl acetate/acetic acid (10/1, by volume) and 0.5 mL of 3 M potassium acetate. In the second step, 250μL of η-butanol and 1.9 mL of 3M potassium acetate in 1mol/L KOH was added to 625μL of the upper (organic) phase isolated in step 1 and vortexed. In the final step, 1.5mL of 2 M orthophosporic acid (H3PO4) and acetic acid (9/1, by vol) was added to 250μL of the upper phase removed from step 2. The amount of porphyrin was quantified using a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Excitation and emission wavelengths were set at 402 nm and 653 nm respectively, and a nonfluorescing yellow Corning filter was added to ensure that blue and violet light were excluded. Wavelength slits were set at 20 nm. Intact heme iron was calculated as the difference in porphyrin values between the two solutions.

2.4.5 Lipid peroxidation

TBARS was measured in the cecal and fecal water following the method of Sesink et al (1999). In brief, 300 μL of diluted fecal water (1:10 with milliQ water) was added to 300 μL of 8.1% lauryl sulphate and 3 mL of 0.5% 2-thiobarbituric acid (TBA) in 10% acetic acid (pH 3.5). The solution was heated for 60 min at 95°C. TBARS were then extracted using 3.6 mL n-butyl alcohol. Samples were centrifuge at 4000 rpm for 10 min, the top layer was removed and placed in a cuvette for analysis on a florescent spectrophotometer at an absorbance of 532 nm.

Urinary MDA was determined following the method as described in Draper et al (1984). In brief, 1mL of urine was added to 4mL of saturated TBA solution in phosphate buffer. The pH was adjusted to 2.8 ± 0.1 by adding HCl and the solution was heated in a boiling water bath for 30 min. The solution was then cooled to room temperature, before being filtered using a Sep Pak cartridge (C18, 500mg, 6mL Supelco/Alltech). The Sep Pak cartridge was first rinsed with 10 mL of methanol, before the cooled solution was added. The cartridge was then rinsed with 15 mL of milliQ water and allowed to dry. MDA was eluted by adding 4mL methanol to the cartridge. The eluted MDA-methanol solution was dried under a stream of air at 70°C, the residue was dissolved in 2 mL of milliQ water and a 100μl aliquot was analysed by High Performance Liquid Chromatography.

The HPLC system used was from Shimadzu (Shimadzu Corporation, Kyoto, Japan). The system was equipped with a pump (Shimadzu, LC10AT), diode array detector (Shimadzu, SPD-MAVP) and an autoinjector (Shimadzu, SIL-10A). The analysis of chromatographs was performed with Shimadzu Class LC10 software. The mobile phase was 17% methanol in MilliQ water, 15% acetonitrile and 0.6% tetrahydrofuran in 5mM phosphate buffer (pH 7). The column was a μBondpack C18 stainless steel analytical column 0.39 x 30 cm and was attached to a 3 x 22-mm guard column packed with Bondapack C18/Corasil (Waters). The detector was set at 546 nm and the flow rate was 2 mL/min.

2.4.6 Fecal fat

Fat was extracted from the feces by acidified chloroform:methanol extraction as described by Folch et al (1957). In brief, a 100 mg sample of freeze dried feces was

placed into a tube and 5 mL of 25% HCl was added. The tube was vortexed thoroughly and 10 mL chloroform and 5 mL methanol was added. The tube was vortexed and then placed on a rotator for 2 h. The tube was then centrifuged at 1000 g for 10 min. The chloroform layer was removed and placed in a pre-weighed test tube. A further 5 mL of chloroform was added to the fecal pellet and mixed on the rotator for a further 1 h. The tube was centrifuged at 1000 g for 10 min, the chloroform layer was removed and combined with the first extract. The chloroform extract was dried under nitrogen at 60°C. The weight of the extracted fecal fat was divided by the weight of the freeze dried fecal sample and expressed as g fat per 100g of freeze dried feces.

2.4.7 Fecal protein

Fecal protein was quantified by the Dumas method (Kirsten and Hesselius, 1983) using a Carlo Erba NA 1500 nitrogen analyzer (Milan, Italy). Samples were prepared for analysis in triplicate, by placing 5 ± 0.5 mg of ground, freeze dried feces into lead cups.

2.4.8 Fecal minerals

Fecal minerals (sodium, potassium and calcium) were quantified using an Atomic Absorption Spectrometer (Varian, Victoria, Australia). Freeze dried fecal samples (100 mg) were prepared for analysis by an over night digestion in 3 mL nitric acid and 200 μL perchloric acid and heated at 110°C. The temperature was then increased to 130°C for 1 h, 150°C for another hour and increased to 170°C until most of the organic residue was gone. The samples were then diluted appropriately with milliQ water and analysed.

2.5 General Lipid measures

2.5.1 Body composition analysis

The epididymal and retroperitoneal fat pads were removed from the rats by gross dissection and weighed. The remaining internal organs, skin, feet, head and tail were also removed leaving the muscle mass and skeleton which was weighed and classified as carcass. The carcass was dried and minced to obtain a consistent sample for fat analysis.

2.5.2 Plasma analysis

The plasma insulin concentration was determined by radioimmunoassay (Linco Research, Missouri, USA). Plasma glucose and triglycerides were determined spectrophotometrically using commercially available kits (Gluco-quant®, Roche, Indianapolis, USA, Triglycerides GPO-PAP, Roche, Indianapolis, USA). Plasma free fatty acid was measured by an enzymatic fluormetric method (Roche, Mannheim, Germany). Plasma IGF-I was measured by ELISA (Immunodiagnostic Systems, Boldon, UK). Thyroid hormones, T3 and T4 were measured by radioimmunoassay (Brahms, Hennigsdorf, Germany). Insulin resistance was quantified by using the insulin to glucose ratio which has been shown to correlate strongly with measures obtained from the euglycemic-hyperinsulinemic clamp technique (Ahren and Larsson, 2002).

Chapter 3 – EFFECT OF DIETARY FATS AND HEME ON RISK MARKERS FOR COLON CANCER

3.1 Introduction

Colon cancer is the second most common cause of death from cancer in Western countries. There is a wide geographic variation in incidence, with a 20-fold variance world wide (Potter, 1996). Population based epidemiological studies have shown that when people migrate from low-risk to high-risk areas, colon cancer increases accordingly to match the host population (Bingham et al, 1996). Although it is recognised that genetic factors are important determinants for the genesis of colorectal cancer in individuals, environmental factors, such as diet composition, may account for up to 70% of colon cancer causation (Doll and Peto, 1981; Willett, 1995). The World Cancer Research Fund (WCRF, 1997) concluded that diets high in vegetables decrease the risk of colon cancer, whereas alcohol and red meat, but not white meat, probably increase risk. It is not known why red meat consumption may increase colon cancer risk more than white meat but possible factors include: brownness of meat (heterocyclic amines), N-nitroso-compounds, heme iron and fat type.

Babbs (Babbs, 1990) hypothesised that high amounts of unabsorbed iron resulting from excessive dietary iron may catalyse the production of oxygen radicals, thus increasing fecal mutagenicity, activating carcinogens or forming tumor promoters within the intestinal lumen. However, there is mixed evidence of an association between high dietary iron intakes and iron status with colon cancer risk (Nelson, 1992). The variation in results may be due to the form of iron ingested. Sesink et al (1999) investigated the effect of different dietary iron sources in Wistar rats on risk markers of colon cancer. Heme iron, in comparison to ferric citrate, protoporphyrin IX and bilirubin, produced a

highly cytotoxic environment in the colon as shown by an increase in cell proliferation, lipid peroxidation and fecal water cytotoxicity. Furthermore, Pierre et al (2003) showed that dietary heme iron (hemin and hemoglobin) increased the number of ACF in the colons of AOM treated F344 rats. Dietary heme iron, as hemoglobin, also increased the incidence of colon tumors in female F334 rats administered intrarectally with *N*-nitroso-*N*-methylurea (Sawa et al, 1998).

The type of dietary fat may be an important factor affecting the iron induced generation of radical species from fat in the colon. The presence of two or more double bonds within polyunsaturated fatty acids increases their susceptibility to oxidation in comparison to saturated fatty acids containing no double bonds. A study by Chin and Carpenter (1997) investigated the interaction between non-heme iron (ferrous fumerate) and fat type (corn oil, menhaden oil and beef tallow) and its effect on risk markers for colon cancer that included colonocyte proliferation and lipid peroxidation in the feces and colon mucosa. Although colonocyte proliferation was unaffected by diet, comparing the high iron diets, rats fed corn oil had significantly higher peroxidised lipids in the feces compared to the beef fed rats. A trend was also shown for increased peroxidised lipid in the mucosa of the rats fed high iron and corn oil compared to the high iron and beef fed rats. This suggests that dietary iron oxidises polyunsaturated fat in the colon and may also exert damaging effects to the mucosa. However, it is not known whether a similar effect would be seen if heme iron was used rather than a non-heme iron source.

Milk fat with a high content of saturated fat is a good source of conjugated linoleic acid (CLA), which has been shown to protect against colon cancer (Liew et al, 1995; Parodi,

1997; Yang et al, 2001). CLA has been shown to be an effective antioxidant *in vitro* (Ha et al, 1990) and when fed to animals it reduced the amount of TBARS in mammary tissue (Ip et al, 1991). CLA has also been shown to protect against IQ-induced adduct formation in the rat (Liew et al, 1995). Therefore, in a diet containing heme iron, milk fat may act to protect the colon from potential pro-oxidant effects of heme-iron.

It was hypothesized that the addition of heme to a diet containing SSO, but not milk fat would increase risk of colon carcinogenesis. The aims were to investigate the interactions between heme and fat in the colon by examining 1) the cytotoxicity of cecal and fecal water on colon cancer cells in vitro, 2) the rate of colonic epithelial proliferation and 3) lipid peroxidation in the cecal contents, feces and urine.

3.2 Experimental design

3.2.1 Animals and diets

Sixty 55-day-old outbred Sprague-Dawley rats were purchased from the Animal Research Centre at Murdoch University (Perth, Western Australia) and separated randomly into 6 groups of 10 animals. Animals were housed in wire cages to minimize coprophagy and maintained in an air-conditioned environment of 23 ± 2°C with a 12:12-h light-dark cycle. They were given free access to diet and water. Fat was added to diets and refreshed daily. After 3 weeks on experimental diets, each rat was placed in a metabolism cage. Diet intake was recorded and fecal and urine samples were collected. At the conclusion of the experiment the rats were euthanased and blood was removed by abdominal aorta exsanguination. Two centimeter sections were removed from the distal

colon and stored in 10% formol-saline. The contents of the cecum were removed, weighed and frozen. All experimental procedures using animals were approved by the Commonwealth Scientific and Industrial Research Organisation, Health Sciences and Nutrition Animal Ethics Committee and the University of Adelaide Animal Ethics Committee.

The experimental high fat rodent diets were based on an AIN-93 purified diet with low calcium (0.1%) and low fiber (2%) (**Table 3.1**). Fat quality and heme iron varied between dietary treatment groups. Dietary treatments contained 20% fat as sunflower seed oil (SSO) or anhydrous milk fat (AMF) and heme iron was added at 0, 0.02 or 0.08g/100g diet. SSO was added at 1g/100g to the AMF diets to provide sufficient essential fatty acids. The fatty acid composition of SSO and AMF is shown in **Table 3.2**.

3.2.2 Cecal and fecal water preparation

Fecal water was prepared following the method of Lapre et al (1991) and Sesink et al (1999). Approximately two grams of freeze dried feces was reconstituted with milliQ water to 30% of dry weight. Cecal contents were diluted with isotonic saline (0.5g/3ml) to obtain sufficient sample (Coleman et al, 2002). The samples were vortexed and then mixed in an incubator at 37°C for 1 h and centrifuged at 26,000 g for 30 min. The supernatant was removed and the osmolarity checked. If the osmolarity differed from 300 mOsM further water was added to another sample of freeze dried feces to obtain an osmolarity of 300 mOsM. Fecal water was stored at -20°C until analysis.

Cecal and fecal water was sterile filtered using a milli-pore filter (0.5 μm) before use.

3.2.3 Cytotoxicity assay

The cytotoxic effect of cecal and fecal water was quantified using HT-29 cells following the method outlined in section 2.4.3. To compare the cytotoxicity of cecal and fecal water samples from different dietary treatment groups, the concentration at which the cecal or fecal water killed 50% of the cells was calculated (IC-50 value) (Kirana et al, 2003). To obtain the IC-50 value, cecal and fecal water samples were diluted by 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 using 0.9% saline. Each dilution was assessed using the cytotoxicity assay and the percentage of cells killed was plotted on a graph. The dilution at which 50% of the cells were killed was recorded.

3.2.4 Biochemical assays

Minerals (sodium, potassium and calcium) were extracted from fecal samples by acid extraction and their concentration was quantified by using an Atomic Absorption Spectrometer (AAS, Varian, Victoria, Australia). Fat was extracted from feces by a chloroform/methanol extraction as outlined in the method of Folch et al (1957). The amount of intact heme in the feces was measured by the Hemoquant assay (Schwartz et al, 1983) using hemin as the standard. TBARS was measured in the cecal and fecal water following the method of Sesink et al (1999). Urinary TBARS was determined by the method described in Draper et al (1984).

3.2.5 Colon epithelial proliferation

Sections of distal colon were processed, mounted in wax blocks and 4 μm sections were cut using a Leitz microtome. Slides were dewaxed, rehydrated and then incubated in 3% hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity. Sections were

then hydrolysed in 2N HCl for 30 min and incubated in 10% normal horse serum (Vector, California, USA) for a further 30 min. Monoclonal murine anti-PCNA antibody PC-10 antibody diluted 1/1000 (Neomarkers, California, USA) was then applied to sections and incubated for 90 min. A negative control, tris buffered saline (TBS) was applied to one section instead of the anti-PCNA antibody. Following a rinse in TBS, the sections were incubated with biotinylated antimouse IgG (Vector, California, USA) for 30 min. Sections were then rinsed in TBS and incubated with conjugated streptavidin peroxidase diluted 1/1000 (Rockland, Philadelphia, USA), for a further 30 min before being stained with 0.25% 3, 3-diaminobenzidine (Sigma Chemicals, Missouri, USA). Sections were counterstained with Mayers Haematoxylin for 20 seconds prior to dehyradation and coverslipping. Slides were scored by examining 20 complete half crypts. The number of stained and unstained cells in the crypt was recorded. Proliferating cells were reported as a percentage of total cells in the crypt.

3.2.6 Statistics

Statistical analyses were performed using SPSS 10.0 for windows (SPSS Inc, Chicago, USA). Group results were compared using a one-way analysis of variance test followed by a Tukey's multiple comparison test. Regression analysis was used to determine whether there was a relationship between heme concentration and fecal measures or colon histology. Values were expressed as mean ± SE and differences between the treatment means were only considered significant when $p<0.05$.

3.3 Results

Body weights and diet intakes were not significantly different between dietary treatment groups (data not shown).

The addition of 0.02% and 0.08% heme to the SSO diets increased fecal output 2-fold in comparison to the other groups (**Table 3.3**). For animals fed the SSO diets, increasing the concentration of heme was positively associated with the amount of fat and moisture in the feces, along with sodium and potassium concentration ($p<0.05$). However, the addition of heme to the AMF diets did not effect the fecal excretion of fat, sodium or potassium ions. There was a positive association with heme concentration in the AMF diet and fecal moisture ($r=0.88$, $p<0.05$) but this increase was not as large as when heme was added to the SSO diets.

Dietary fat type and heme interactively affected cecal and fecal water cyotoxicity. Undiluted cecal and fecal water from all dietary treatment groups was highly cytotoxic (greater than 95%). Upon dilution, it was possible to obtain the concentration of cecal and fecal water that killed 50% of cells **(Figure 3.1)**. The cecal and fecal water from rats fed the SSO diet in comparison to the AMF diet was less cytotoxic ($p<0.05$). The addition of heme to the sunflower seed oil diet increased cytotoxicity of both cecal and fecal water in a dose responsive manner **(Figure 3.1)**. The increase in cytotoxicity was largest in the fecal water, with 100% cytotoxicity at 1/128 dilution for the SSO groups with heme (0.02% and 0.08%). In contrast, the addition of 0.02% and 0.08% heme iron to the AMF diets reduced the cytotoxicity of the cecal water and had no effect on fecal water cytotoxicity.

Dietary heme or fat type did not effect the number of proliferating cells in the colon (**Table 3.4**). However, the addition of 0.02% and 0.08% heme to the SSO diets increased crypt height by 24% ($p<0.05$) (**Table 3.4**). This effect of heme on crypt height was not observable with the AMF diets.

The amount of TBARS in the cecal and fecal water and urine was affected by heme concentration but not fat type (**Table 3.5**). The addition of heme to SSO and AMF diets correlated with TBARS formation in cecal and fecal water and urine. TBARS was greater in the fecal water than in the cecal water consistently for all dietary treatments.

3.4 Discussion

Recent evidence suggests that heme iron from red meat may be a promotional factor of colon carcinogenesis. However, it was not clear how heme iron in the colon may interact with different fat types to influence colon cancer risk. The current study showed that the addition of heme to the SSO diet increased the cytotoxicity of cecal water and produced a fecal water that was highly cytotoxic. Previous studies have shown that when heme iron was added to diets containing 20% fat (mixture of palm oil and corn oil) cytotoxicity increased but not to the level seen in the current study (Sesink, 2000; Sesink et al, 2000). The difference between these studies may be due to the different types of fats used and suggests that a diet containing heme iron and high fat predominantly comprised of polyunsaturated fat may be more effective in producing highly cytotoxic fecal water. Previous studies have shown that a heme-induced increase in fecal water cytotoxicity was

related to increased colonic proliferation (Sesink, 2000; Sesink et al, 2000). Although an increase in proliferation was not seen in the current study, an increase in the height of the crypts was seen only when heme iron was added to SSO based diets. This suggests that the heme induced changes in the fecal composition may alter cell cycle regulation. As a significant change was not seen in proliferation rate it does suggest that the longer crypts may be caused by a reduction in the rate of apoptosis. As apoptosis is the process whereby cells with DNA damage are removed or deleted, a reduction in apoptosis is related to an increased risk of colon cancer (Bedi et al, 1995). In the current study apoptosis was not measured, as the number of apoptotic cells in control rats was very low (1 cell per 10 crypts) which made it difficult to detect changes in apoptosis between treatment groups (Hu et al, 2002). Therefore a follow up study is required using an animal model in which apoptosis rates are higher or stimulated by an agent such as a carcinogen.

The cytotoxic substance in fecal water of rats consuming the SSO diet with heme iron is unknown. Sesink (2000) described the cytotoxic substance as being a hydrophobic, pronase-resistant polypeptide in the lipid phase of fecal water, which may be produced by bacteria in the colon. Another possible explanation is that the cytotoxic substance is a radical species produced from the oxidation of fat in the presence of iron. As SSO is more vulnerable to oxidation than other fats, this may account for the highly cytotoxic fecal water seen in the current study when heme was added to the SSO diets. Chin and Carpenter (1997) showed that in a diet containing non-heme iron, the addition of corn oil increased the concentration of TBARS in feces in comparison to beef fat. Furthermore, when heme was incubated with different fat types it was the oxidized, refined vegetable

oils, particularly safflower oil, that readily generated lipid peroxyl radicals in the presence of various heme compounds, and this did not occur with unpurified native vegetable oils (Sawa et al, 1998). In the current study, TBARS concentration in cecal contents, feces and urine were not affected by fat type. These findings suggest that although milk fat is oxidized in the presence of heme, it may not be producing the same radical species as the SSO based diets.

The milk fat based diets did not change the amount of fecal fat or cations in the feces, the proliferating index of colonic epithelial cells or crypt height. Furthermore, the addition of heme to the AMF diets reduced cecal water cytotoxicity. These findings suggest that AMF inhibited the damaging affects of heme iron when added to the diet at 0.02% and 0.08%. The way in which AMF may be reducing the heme induced cytotoxic effect (as seen with SSO) may be due to the different ways these fats are oxidised in the presence of heme (as described previously), or it may be due to specific components found in AMF, such as CLA, butyrate, sphingolipids.

The antioxidant effects of CLA as shown previously in vitro (Ha et al, 1990) were not evident in the AMF fed rats as TBARS in cecal and fecal water and urine increased with increasing heme iron in the diet. The concentration of TBARS also increased to a similar level as seen in the SSO diets. There were no changes in cell proliferation and as apoptosis was not measured in this study we can not determine whether sphingolipid or butyrate in AMF were in sufficient concentration to effect cell cycle regulation in the colon.

These findings suggest that the cytotoxic effect of heme iron in the colon may be modified by fat type. The generation of lipid peroxides by heme seems to be more complicated than measuring net peroxide generation. AMF was shown to inhibit the heme induced changes in feces as seen with SSO. However, it is not clear whether this effect was specific to AMF or whether it is a saturated fat effect. Therefore, it is important to investigate whether the effect seen in this study is specific to AMF or is typical of all saturated fats such as a meat fat like tallow. This study highlights the important role of fat type particularly in a diet containing a high quantity of heme iron on colon cancer risk.

Table 3.1. Composition of experimental diets based on AIN-93 formulation

Components	SSO	SSO +0.02% heme	SSO +0.08% heme	AMF	AMF +0.02% heme	AMF +0.08% heme
Casein	20	20	20	20	20	20
Sugar	20	20	20	20	20	20
Cornstarch	33	33	33	33	33	33
Fiber	2	2	2	2	2	2
Fat						
Sunflower seed oil	20	20	20	1	1	1
Anhydrous milk fat	0	0	0	19	19	19
Heme	0	0.02	0.08	0	0.02	0.08
Cholesterol	0.49	0.49	0.49	0	0	0
Choline	0.2	0.2	0.2	0.2	0.2	0.2
Cysteine	0.3	0.3	0.3	0.3	0.3	0.3
Minerals	3.5	3.5	3.5	3.5	3.5	3.5
Vitamins	1	1	1	1	1	1

Values are expressed as g/100g diet, SSO, sunflower seed oil, AMF, anhydrous milk fat, AIN-93 formulation is described in Reeves et al (Reeves et al, 1993).

Table 3.2. Composition of sunflower seed oil and anhydrous milk fat

	Sunflower seed oil	Anhydrous milk fat
Saturated	12.7	67.8
Mono-unsaturated	30	28.6
Poly-unsaturated	57.3	3.6
Conjugated linoleic acid	0.05	0.88

Values are expressed as g/100g of lipid.

Table 3.3. Effect of diets on feces composition

	Fecal output/ day (wet)	Moisture (%)	Fat (mg/day)	Heme iron (μmol/day)	Sodium (μmol /day)	K+ (μmol/day)	Ca+ (μmol/day)
SSO	0.9 ± 0.05^{a}	53 ± 0.3^{a}	60 ± 3^{a}	0.1 ± 0.02^{a}	69 ± 9^{a}	67 ± 14^{a}	40 ± 9
SSO + 0.02% heme	2.0 ± 0.1^{b}	70 ± 0.4^{b}	88 ± 5^{b}	1.2 ± 0.1^{b}	213 ± 21^{b}	163 ± 11^{b}	24 ± 6
SSO + 0.08% heme	2.0 ± 0.1^{b}	77 ± 1.9^{b}	111 ± 8^{c}	4.0 ± 0.5^{c}	209 ± 42^{b}	178 ± 35^{b}	25 ± 3
AMF	1.1 ± 0.07^{a}	53 ± 2.7^{a}	59 ± 5^{a}	0.2 ± 0.01^{a}	61 ± 7^{a}	41 ± 16^{a}	27 ± 5
AMF +0.02% heme	1.1 ± 0.03^{a}	57 ± 3.9^{a}	54 ± 2^{a}	1.78 ± 0.3^{b}	64 ± 3^{a}	42 ± 8^{a}	52 ± 13
AMF +0.08% heme	1.1 ± 0.04^{a}	66 ± 0.3^{a}	58 ± 1^{a}	4.8 ± 0.2^{c}	57 ± 9^{a}	25 ± 3^{a}	31 ± 6
Correlations							
SSO	0.76, 0.001	0.95, 0.01	0.9, 0.001	0.89, 0.007	0.66, 0.007	0.67, 0.006	ns
AMF	ns	0.88, 0.02	ns	0.95, 0.001	ns	ns	ns

Different superscript letter denotes significant difference between dietary treatment group with in each column ($p<0.05$). SSO, sunflower seed oil, AMF, anhydrous milk fat. Correlation data is presented as r value, p value. ns; no significance.

Table 3.4. Effect of diets on crypt height and the number of proliferating cells in the distal colon

	Labeling index[1] (%)	Height of crypt (cells/half crypt)
SSO	33 ± 1.5	29 ± 0.7 [a]
SSO + 0.02% heme	37 ± 2.2	38 ± 0.9 [b]
SSO + 0.08% heme	39 ± 2.6	38 ± 1.1 [b]
AMF	34 ± 1.5	29 ± 0.6 [a]
AMF + 0.02% heme	37 ± 1.5	30 ± 0.9 [a]
AMF + 0.08% heme	39 ± 3.8	30 ± 0.4 [a]
Correlations		
SSO	ns	0.79, 0.001
AMF	ns	ns

[1] labeling index refers to the percentage of PCNA positive cells in the crypt. Different letters between group in each column denotes significant difference ($p<0.05$). SSO, sunflower seed oil, AMF, anhydrous milk fat. Correlation data is presented as r value, p value. ns; no significance.

Table 3.5. Effect of diets on the concentration of TBARS in cecal water, fecal water and urine

	Cecal water (nmol MDA/ml)	Fecal water (µmol MDA /ml)	Urine (nmol MDA/ day)
SSO	9 ± 2.3[a]	27 ± 3.4[ab]	10 ± 2.1[a]
SSO + 0.02% heme	12 ± 2.3[abc]	42 ± 4.9[abc]	29 ± 4.3[ab]
SSO + 0.08% heme	21 ± 2.1[c]	66 ± 9.5[b]	49 ± 8.8[b]
AMF	12 ± 2.0[b]	19 ± 2.2[a]	13 ± 2.5[ac]
AMF + 0.02% heme	16 ± 2.8[abc]	44 ± 6.8[abc]	33 ± 5.5[ab]
AMF + 0.08% heme	19 ± 1.9[c]	76 ± 9.5[c]	35 ± 7.5[bc]
Correlation			
SSO	0.72, 0.001	0.67, 0.006	0.71, 0.001
AMF	0.49, 0.023	0.74, 0.002	0.48, 0.011
ANOVA			
Fat type	ns	ns	ns
Heme	0.001	0.001	0.001
Fat * heme	ns	ns	ns

TBARS is reported as malondialdehyde equivalents (MDA). A different letter between groups in each column denotes significant difference ($p<0.05$). SSO, sunflower seed oil, AMF, anhydrous milk fat. Correlation data is presented as r value, p value. ns; no significance. ANOVA, p value shown if there is a significant effect.

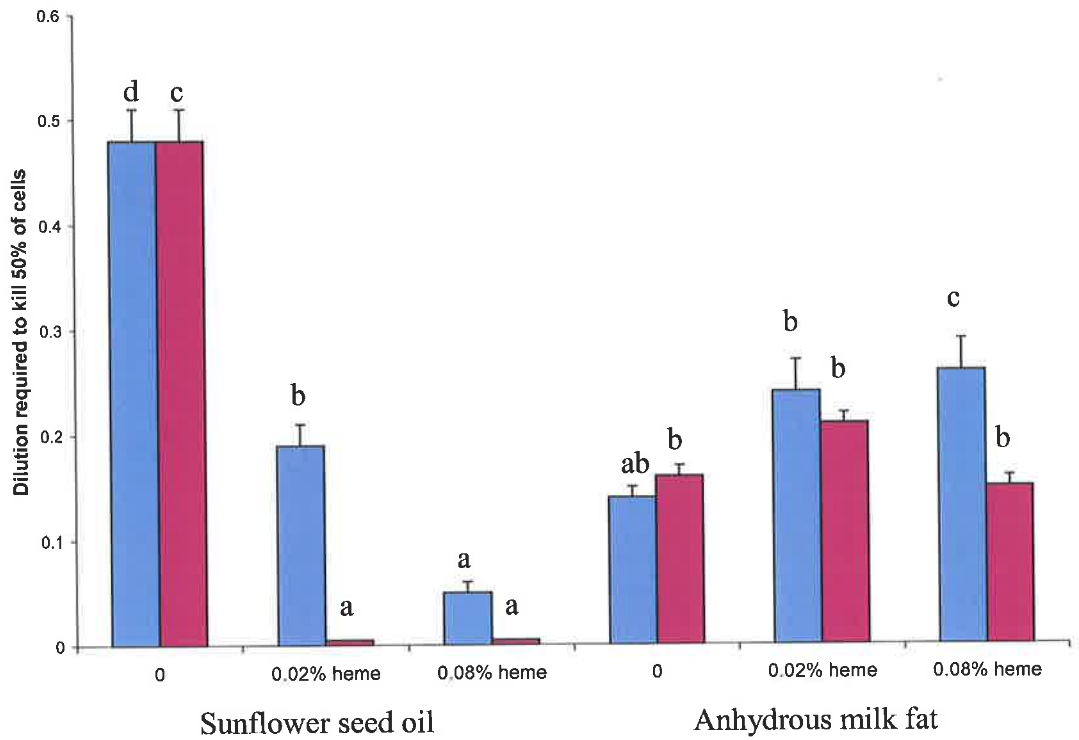


Figure 3.1. Concentration of cecal and fecal water that killed 50% of HT-29 cells in the cytotoxicity assay. A lower value indicates greater cytotoxicity. Different superscript letter denotes significant difference between dietary treatment group for cecal water (■) and fecal water cytotoxicity (■) ($p<0.05$). Fecal water from rats fed 0.02% and 0.08% heme iron and sunflower seed oil diets were highly cytotoxic even at 1/128 times dilution.

Chapter 4 – GENOTOXIC EFFECTS OF DIETARY HEME ON RAT COLONOCYTES IN VIVO

4.1 Introduction

Western countries including America, Europe and Australia have the highest incidence of colon cancer, accounting for nearly two thirds of worldwide incidence (Potter, 1996). Although it is recognised that genetic factors are important determinants in the development of colon cancer in individuals, environmental factors, such as diet and diet composition, may account for up to 70% of colon cancer causation (Doll and Peto, 1981; Willett, 1995). Typical Western diets, high in total fat, animal fat, red meat and low in vegetables, have been associated with an high incidence of sporadic cancers (colon, breast and prostate) (WCRF, 1997).

The high red meat component of the Western diet has been shown to increase colon cancer risk by up to 2.5 times (Willett et al, 1990). A recent meta-analysis showed that for every 100g of red meat consumed per day, colorectal cancer risk increased by 12-17% (Sandhu et al, 2001). Several components of red meat have been postulated as being involved in increasing colon cancer risk, including heme iron.

Dietary heme iron is considered to have potential to increase colon cancer risk as significant quantities of heme ingested from red meat (25%) reached the colon (Schwartz and Ellefson, 1985). Once in the colon, heme has the potential to act as a highly pro-oxidant molecule to generate radical species that may damage the DNA of colonocytes. Animal studies have shown that feeding rats a diet high in heme iron, as hemin or hemoglobin increased colon cancer risk (Sawa et al, 1998; Sesink et al, 1999; Pierre et al, 2003). When hemin was added to a control diet it increased fecal water TBARS,

cytotoxicty and proliferation of the colon epithelium (Sesink et al, 1999). When these animals are injected with a carcinogen (azoxymethane), dietary hemin increased ACF number and size by up to 4 fold (Pierre et al, 2003). Similarly, the addition of hemoglobin to a control diet increased ACF number in AOM treated rats (Pierre et al, 2003) and TBARS in fecal water, but did not affect fecal water cytotoxicity. Hemoglobin also increased the incidence of NMU-induced tumors by 2 fold, in comparison to control fed rats (Sawa et al, 1998).

It is not clear what mechanisms are responsible for the promotional effect of heme iron on colon carcinogenesis. Sesink (2000) isolated a cytotoxic substance from the fecal water of rats fed heme iron and identified it as a hydrophobic, pronase-resistant polypeptide in the lipid phase of fecal water, possibly produced by bacteria in the colon. As this substance was not found in the fecal water of rats fed hemoglobin (Pierre et al, 2003), this implies that heme iron may increase colon cancer risk by more than one mechanism. An alternative mechanism might be that heme iron produces a radical species from the oxidation of fat in the digestive tract that can damage the DNA of colonocytes. *In vitro* studies have shown that heme iron compounds (myoglobin, hemoglobin, cytochrome c, hemin and hematin) react with oxidized polyunsaturated fats to generate the lipid alkylperoxyl radical (ROO). This radical species has been shown to exert strong cytotoxic effects on bacterial cells (Akaike et al, 1992; Akaike et al, 1995), induced leakage of potassium ions and impaired enzyme activity in red blood cells (Van der Zee et al, 1989). Furthermore, the peroxyl radical has been shown to cleave plasmid DNA (Sawa et al, 1998) and produce abasic sites in calf thymus DNA (Kanazawa et al, 2000). As the lipid peroxyl radical has a long half life of more than 30 min this gives it sufficient

time to pass into colonocytes to induce genetic damage (Peak and Peak, 1990; Akaike et al, 1992).

Although consistent cytotoxic and genotoxic effects have been found for the lipid peroxyl radical generated from the incubation of heme iron and polyunsaturated fat in vitro, it is not clear whether this genotoxic effect can occur in the colon of rats fed a diet containing heme iron and polyunsaturated fat. Therefore, the aim of this study was to establish the optimal method for colonocyte isolation and to assess the degree of genetic damage in colonocytes isolated from rats fed a high polyunsaturated fat diet with or without heme iron.

4.2 Experimental design

4.2.1 Animals and Diets.

Twelve 10 week-old outbred Wistar rats were obtained from the Laboratory Animal Services Branch, Adelaide University. Animals were housed in wire cages to minimize coprophagy and maintained in an air-conditioned environment of 23 ± 2°C with a 12-h light/12-h dark cycle. Upon arrival, rats were randomly divided into two dietary treatment groups and started on semi-purified diets with or without heme iron (0.02%). The two semi-purified diets were based on AIN-93 formulation with low fibre (2%), low calcium (0.1%) and high fat (20% sunflower seed oil). The concentration of heme iron was balanced in the diets with ferric citrate. Each day, the diet was refreshed and the fat was added to the diet daily. After a feeding period of three weeks, rats were euthanased by

fluothane/oxygen anaesthesia followed by abdominal aortic exsanguination. The colons were then removed and processed as described below.

4.2.2 Colonocyte isolation

The original method for isolating colonocytes by an enzymatic technique was devised by Pool-Zobel and colleagues (1993). In brief, a 5 cm section of rat colon was rinsed with pre-warmed Hanks buffered salt solution (HBSS) 2 to 3 times for a total of 10 min. The colon was then filled with 2 to 3 mls of digestive buffer (1.22mg proteinase K/ml HBSS) and sutured. The colon was placed in a 50 ml Falcon tube half filled with HBSS and placed on a platform shaker for 30 min at 37°C. The colon was removed and the digestive buffer containing the colonocytes was collected. The suspension was centrifuged for 8 min at 100 g. After removal of the supernatant the cell pellet was resuspended in 5 to 10 mls of RPMI 1640.

4.2.2.1 Variation 1

Aim: To determine whether the addition of collagenase to the digestive buffer was effective in improving the isolation of colonocytes.

The different digestive buffers tested contained 50 U/ml proteinase K as contained in the original method, 50 U/ml collagenase or a combination of both enzymes were used (50 U/ml proteinase K and 50 U/ml collagenase).

4.2.2.2 Variation 2

Aim: To determine whether the use of 1 mM DTT was an effective non-enzymatic method to isolate colonocytes.

This variation was adapted from Homaidan et al (1995). In brief, the colon was first rinsed with an isotonic saline solution containing 1 mM DTT. The colon was then everted over a sterile pipette and then filled with a saline solution that also contained 1 mM Na_2EDTA, 10 mM HEPES buffer and 10% foetal calf serum. The colon was placed in a Falcon tube filled with 25 ml of the same solution. After a 10 min incubation at 37°C, the solutions were discarded and a solution was added that contained 20 mM Na_2EDTA. The colon was then incubated for a further 50 min at 37°C. The cell suspension was removed and 2.5 ml of 20 mM $CaCl_2$ was added to achieve a final concentration of 2 mM. It was then centrifuged at 380 g for 10 min. The supernatant was removed and the cells were resuspended in DMEM with 10% foetal calf serum.

4.2.2.3 Variation 3

Aim: To determine whether the addition of DTT prior to the digestive buffer improved colonocyte isolation.

A study by Anderson et al (1999) added an extra step to the original method. Prior to adding the digestive buffer, the colon was filled with 40 mM DTT in HBSS at room temperature for 10 min to dispel the mucus. Colonocytes were then liberated using proteinase K digestion (3.7 mg/ml, in calcium and magnesium free HBSS) at 37°C for 30 min.

4.2.3 Checking the effectiveness of the colonocyte isolation procedures tested

Once the colonocyte isolation procedure was completed a 1 cm section of colon was fixed overnight in 10% formol-saline. Colons were then processed using a Leica tissue processor and mounted in wax blocks, sections (4 μm) were cut by a Leitz microtome, stained with hematoxylin and eosin and assessed by light microscopy. The colon section was examined to quantify the degree to which colonoctytes were removed and whether the lamina propria remained intact.

Cell viability was quantified by Trypan blue staining. A 100 μl sample of cell suspension was centrifuged for 8 min at 100 g. The supernatant was removed and the cell pellet was re-suspended in 25 μl of PBS and 25 μl of 0.2% trypan blue. After 10 min the solution was placed on a cytometer and viable and non-viable cells were quantified using a microscope with a 10 x objective.

4.2.4 Using the isolated colonocytes in the Comet assay

Isolated cells were assessed for genetic damage following the Comet assay method as outlined in chapter 2.3.3. The data is presented as tail length and tail moment. Tail length increases when there are smaller and more numerous DNA strand breaks. Tail moment is a product of tail length and tail intensity. This is considered a more accurate measure of genotoxicity as it also incorporates tail length with the density of the DNA fragments in the Comet tail.

4.2.5 Statistics.

Statistical analyses were performed using SPSS 10.0 for windows (SPSS Inc, Chicago, USA). Results were compared using a one-way analysis of variance, with a Tukey's post hoc test to test for significant differences between means. Values were expressed as mean and standard error of the mean and differences between the treatment means were only considered significant when $p<0.05$.

4.3 Results

4.3.1 Weight gain and food intake.

The body weights (507 ± 9.4 g heme, 486 ± 12.9 g control) and diet intakes (22 ± 0.6g/day heme, 23 ± 0.6 g/day control) of animals was the same in the two dietary treatments.

4.3.2 Determine the most effective technique for colonocyte isolation

4.3.2.1 Variation 1

Incubation of the colons with 50 U/ml protease was partially effective in isolating the colonocytes from the colon crypts (**Figure 4.1**). **Figure 4.1** shows evidence for the isolation of entire crypts (arrow). However, a majority of the crypts remained largely intact (*).

Incubation of the colons with 50 U/ml collagenase was not effective in isolating the colonocytes from the colon wall with most crypts fully intact (**Figure 4.2**).

When the colons were exposed to an enzyme buffer containing collagenase and protease colonocytes were only partially isolated (**Figure 4.3**). This was not different to the isolation seen with protease alone (**Figure 4.1**).

Colonocytes isolated using protease or protease with collagenase showed good viability with 87 ± 4.4% and 89 ± 1.6% respectively. Due to the ineffectiveness of collagenase in isolating cells there were not enough cells to assess viability.

4.3.2.2 Variation 2

The enzymatic method using DTT was effective at isolating the colonocytes from the colon crypts. Incubating cells with DTT for 25 or 50 min did not effect the isolation of colonocytes (**Figure 4.4**a and 4b). A majority of the crypts were removed, some of which can be seen partially intact in the lumen (**Figure 4.4**a). The lamina propria remained intact which suggests the specificity of this technique to the isolation of the colonocytes.

4.3.2.3 Variation 3

The combination of both the non-enzymatic technique and enzymatic technique showed a very efficient isolation of colonocytes from the colon epithelium after a 30 min incubation with protease K, whilst leaving the lamina propria intact (**Figure 4.5**). Viability of cells isolated was also very good, with an average of 87 ± 1.0 % viable cells from the proximal colon and 84 ± 2.2 % of cells viable from the distal colon.

4.3.3 Using the isolated colonocytes in the Comet assay

Figures 4.6 and 4.7 show the effect of 0.02% heme on DNA damage in colonocytes isolated from the proximal and distal colon. DNA damage is represented by tail length (**Figure 4.6**) and tail moment (**Figure 4.7**). In the distal colon, the colonocytes of animals fed the heme diet showed an increase in the amount of genetic damage in comparison to the animals fed the control diet. However, in the proximal colon the DNA of colonocytes isolated was intact and unaffected by dietary intervention.

4.4 Discussion

Previously heme was considered to act as a promoter of carcinogenesis due to its cytotoxic activity (Sesink et al, 1999). However, heme may also act as an initiator of colon carcinogenesis. The current study showed that colonocytes isolated from the distal colon of rats fed heme had more genetic damage in comparison to control fed animals. If the heme induced genetic damage occurs specifically to tumor suppressor genes such as APC, DCC or p53, this may lead to the initiation of cancerous cells.

The generation of radical species by heme is a possible mechanism explaining the increased DNA damage of colonocytes. *In vitro* studies showed that incubated mixtures of heme and polyunsaturated fat were genotoxic to plasmid and calf thymus DNA (Sawa et al, 1998; Kanazawa et al, 2000). This was related to the peroxyl radical that was specifically produced when heme was incubated with highly processed polyunsaturated fats. For this radical to induce damage in colonocytes it must firstly be able to survive and not get mopped up by other substances present in the colon, secondly, pass through the

protective mucous barrier of colonocytes, and thirdly, pass through the cell membrane into the cytoplasm of colonocytes and then into the nucleus where it can then damage the DNA. Previous studies have examined the activity or end products of iron (heme and non-heme iron) on the three steps that are necessary for genetic damage to occur to colonocytes. In the first stage, feces can be collected and the total amount of lipid peroxidation (TBARS, isoprostanes, etc) or the radical generating potential of feces or fecal water can be measured. Heme has been shown to increase the amount of peroxidised lipid (TBARS) in feces and fecal water (Chapter 3) (Sesink et al, 1999). The generation of free radical species in the feces has also been shown to increase with an high concentration of iron in the diet (Lund et al, 1999). In the second stage the mucous layer lining the colon wall can be removed and examined for lipid peroxide generating potential. A study by Lund et al (2001) showed that a high iron diet increased the amount of lipid peroxide generated in the colonic mucosa of rats in comparison to rats fed a low iron diet. This suggests that the mucous layer may not be sufficient to protect the epithelial cells from lipid radicals generated from iron in the colon. In the third stage, the damaging effects of the peroxyl radical can be identified within the cell by assessing cells for lipid peroxidation products (some include MDA, alkanals, 4-hydoxynonenal), DNA-hydroxylation products (8-hydroxy-2'-deoxyguanosine) and/or protein hydroxylation products (ortho-tyrosine, dityrosine). However the effect of dietary heme on the formation of these products in colonocytes is not known.

The current study showed that colonocytes from the distal colon of heme fed rats were more susceptible to genetic damage than colonocytes from the proximal colon. This finding may be related to the difference found in cytotoxicity of cecal and fecal water

isolated from rats fed a high heme diet (Chapter 3). In that study the fecal water of heme iron fed rats killed more HT-29 cells *in vitro* than did cecal water. It is not clear what makes the distal colon more susceptible than the proximal colon to genetic damage but it may be due to the morphological and biochemical differences between these two regions The distal colon in comparison to the proximal colon has a less complex blood supply, larger excretion of acidic mucins, longer crypts, higher rates of apoptosis, less generation of short chain fatty acids and has many differences in xenobiotic detecting enzymes such as P450 (Iacopetta, 2002).

In summary, this study has shown for the first time that dietary heme induced genetic damage to colonocytes isolated from the distal colon. Previously this effect had only been shown to occur in cells in *in vitro* studies using mixtures of heme iron and polyunsaturated fat. However, it is not clear whether the heme induced genotoxicity is due to the generation of peroxyl radicals. Therefore further investigation is required to quantify the amount of peroxyl radicals generated in the feces of heme fed rats. The Comet assay could be modified to include restriction enzymes that would make it possible to determine whether the heme induced genetic damage to colonocytes is due to oxidative damage of DNA.

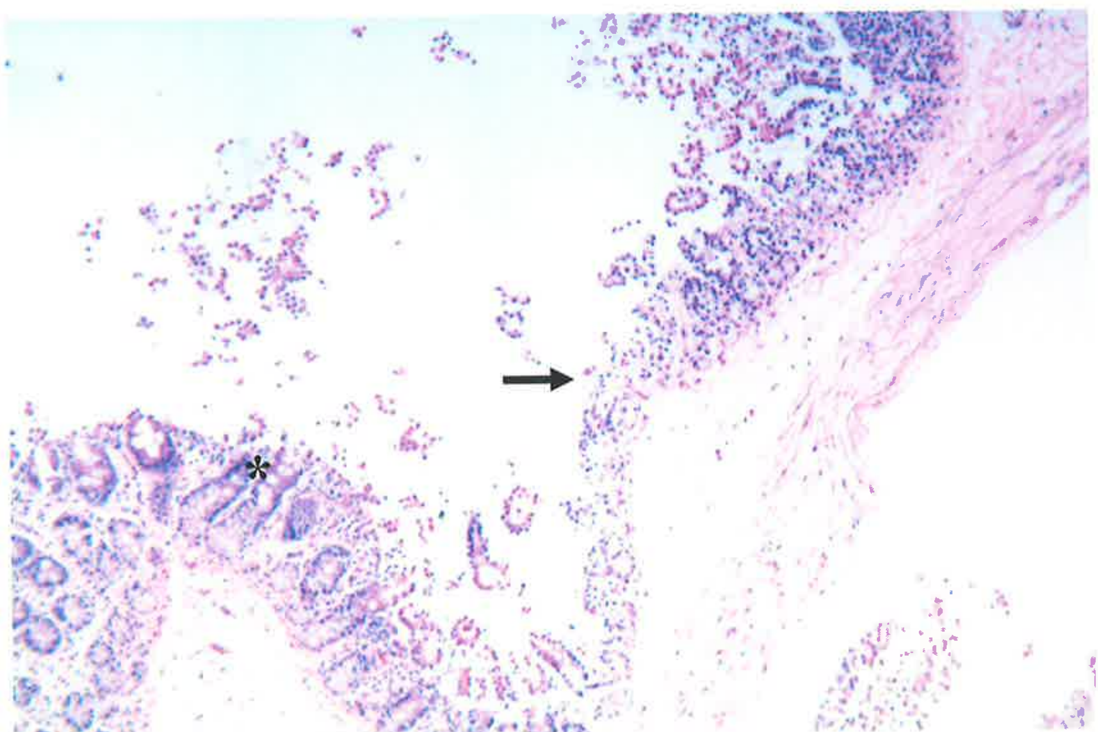


Figure 4.1. A cross section of distal colon treated with 50U/ml protease in the colonocyte isolation procedure (variation 1). The section is stained with haemotoxylin and eosin and is magnified by 100x. This method was partially effective in isolating colonocytes from the crypts. There are regions where colonocyte isolation was effective (arrow) and ineffective (*).

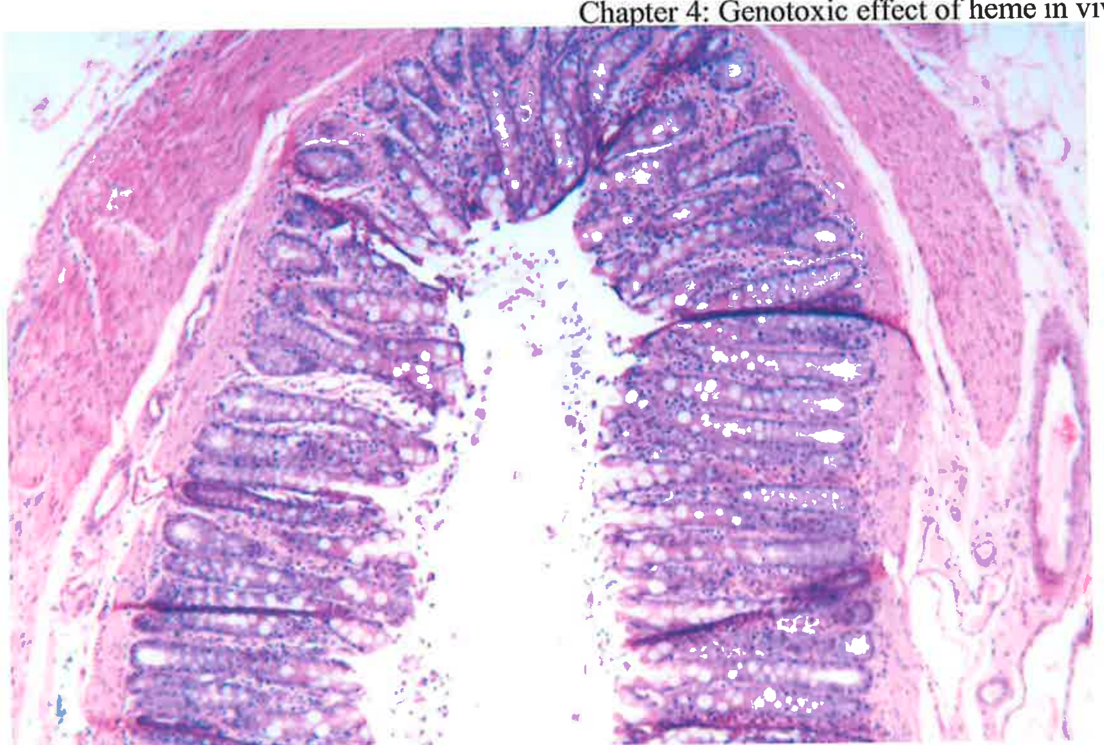


Figure 4.2. A cross section of distal colon treated with 50U/ml collagenase in the colonocyte isolation procedure (variation 1). The section is stained with haemotoxylin and eosin and is magnified by 100x. This method was ineffective in isolating colonocytes as all crypts are intact.

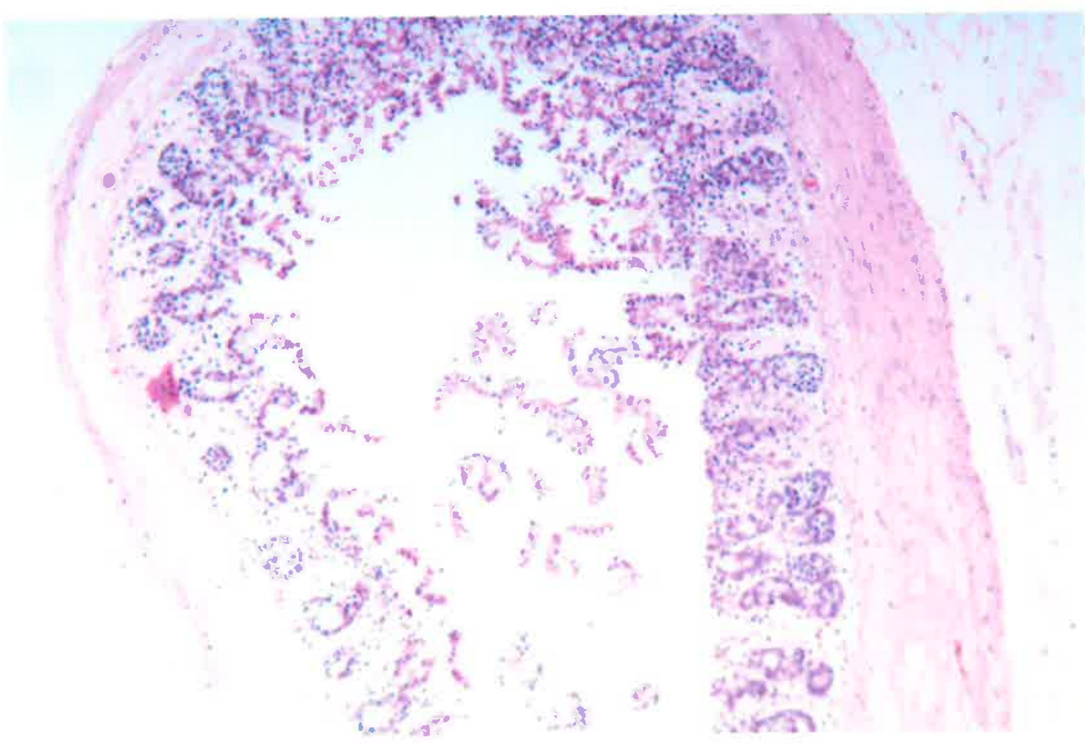
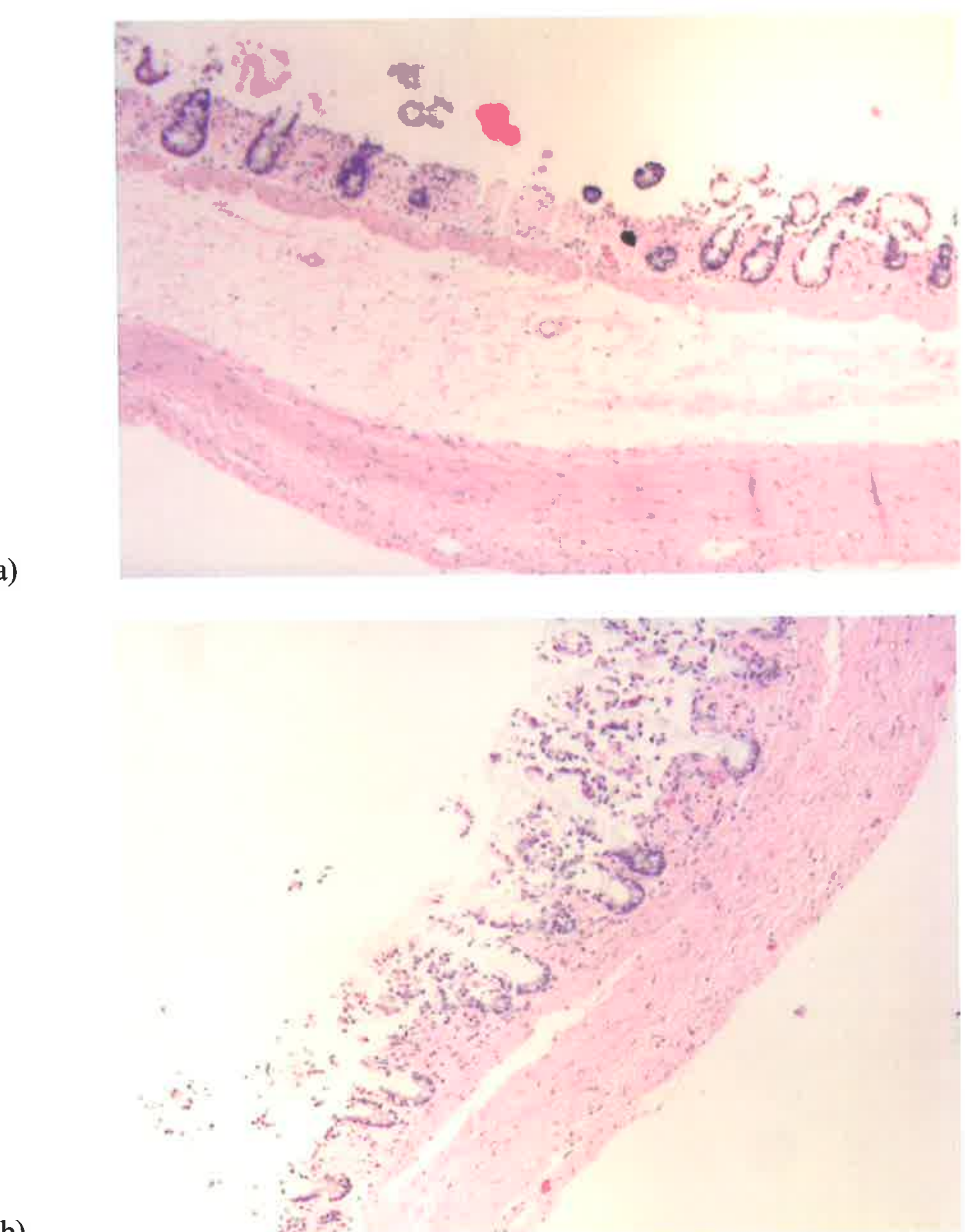


Figure 4.3. A cross section of distal colon treated with 50U/ml protease and 50U/ml collagenase in the colonocyte isolation procedure (variation 1). The section is stained with haemotoxylin and eosin and is magnified by 100x. Combining both enzymes in the digestive buffer did not improve the colonocyte isolation when compared to the use of protease alone (see Figure 4.1).



a)

b)

Figure 4.4. A cross section of distal colon treated with 1mM dithiothreitol in the colonocyte isolation procedure (Variation 2) for 25minutes (a) and 50 minutes (b). These sections were stained with haemotoxylin and eosin and magnified by 100x. This method was partially effective in isolating colonocytes from the crypts. Increasing incubation time from 25 to 50 minutes did not increase the effectiveness of the colonocyte isolation procedure.

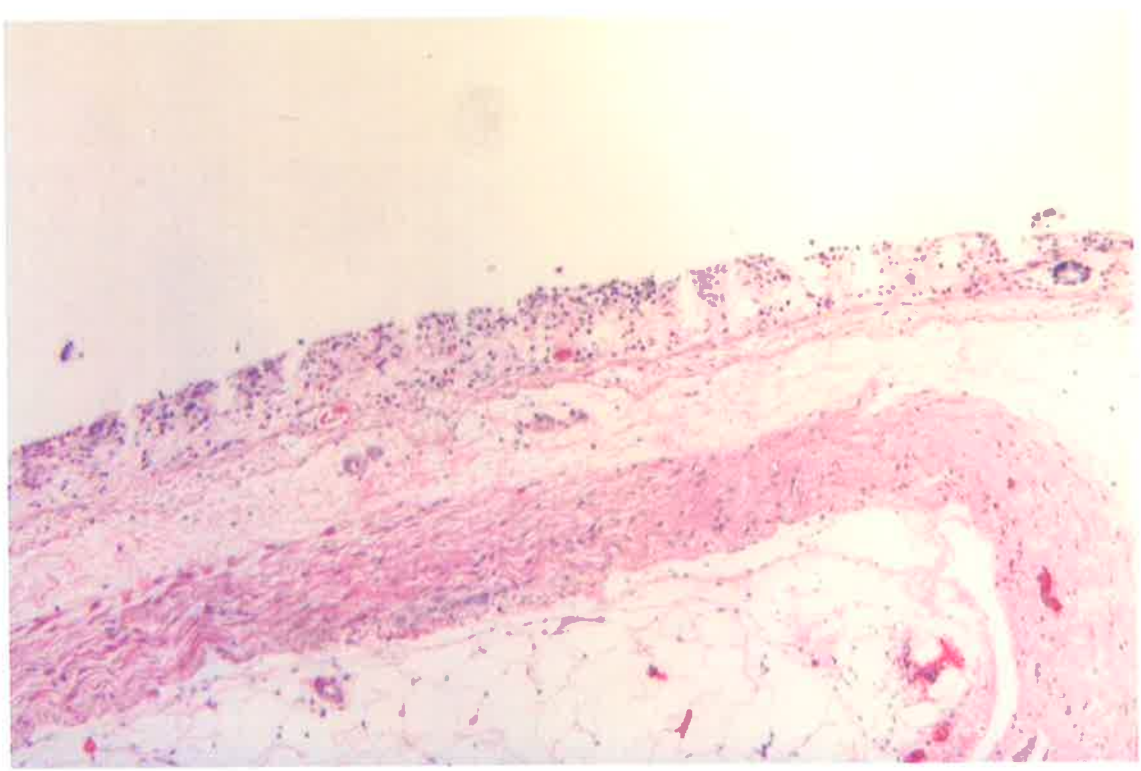


Figure 4.5. A cross section of distal colon treated with 40mM DTT prior to incubation with 50U/ml protease in the colonocyte isolation procedure (Variation 3). This section was stained with haemotoxylin and eosin and magnified by 10x. This method was highly effective in isolating colonocytes from the colon epithelium whilst leaving the lamina propria intact.

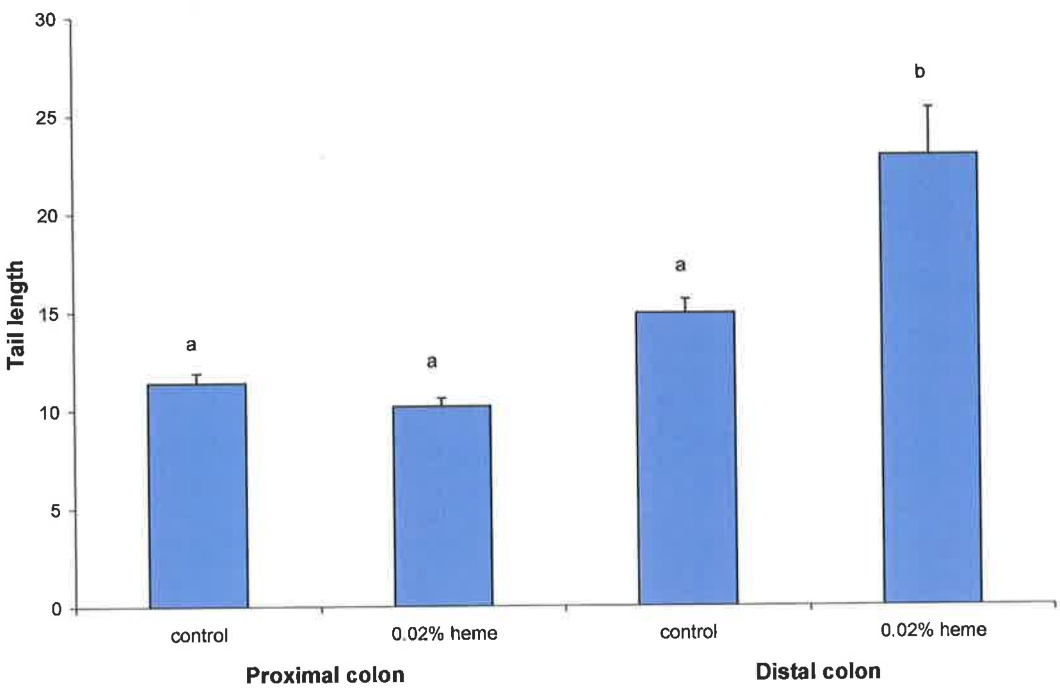


Figure 4.6. The effect of 0.02% heme iron on Comet tail length. Different letters between columns denotes significant difference ($p<0.05$).

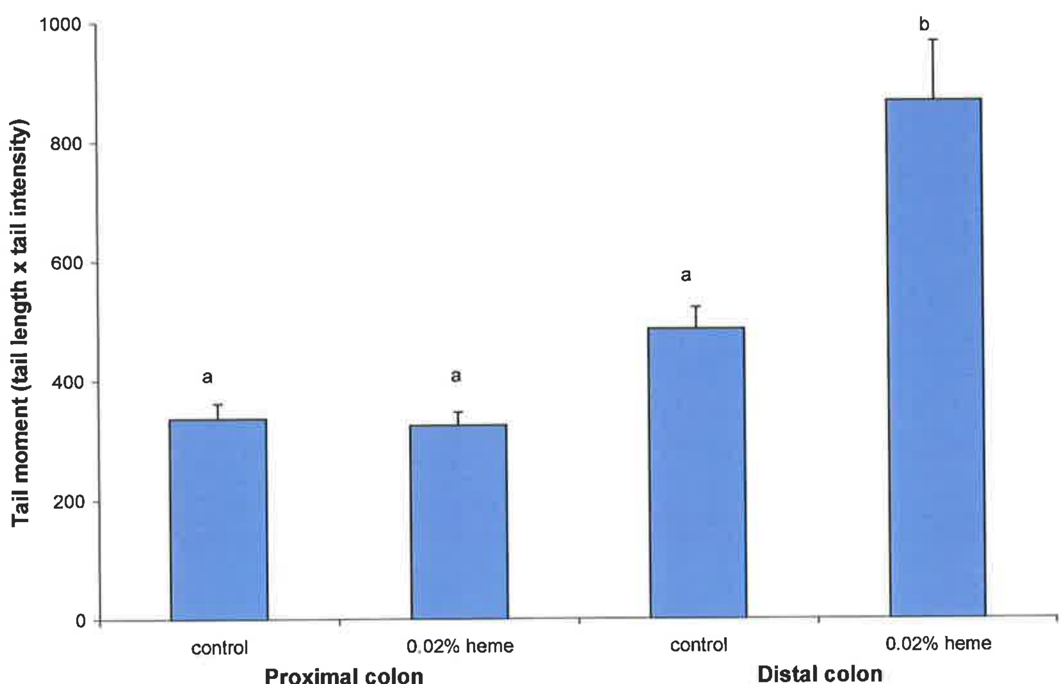


Figure 4.7. The effect of 0.02% heme iron on Comet tail moment. Different letters between columns denotes significant difference ($p<0.05$).

Chapter 5 – WHEY PROTEINS PROTECT MORE THAN RED MEAT AGAINST AZOXYMETHANE INDUCED ACF IN WISTAR RATS

5.1 Introduction

Colorectal cancer is one of the most common cancers affecting men and women in the Western world. Dietary factors could be contributing as much as 70% to causation, and the potential for prevention by dietary means requires further research (Doll and Peto, 1981; Willett, 1995). Epidemiological data generally support the association between total energy intake, high fat diets and red meat intake and increased colon cancer risk (WCRF, 1997). Evidence for red meat is more clearly established than for other dietary protein sources, such as dairy (WCRF, 1997; Yoon et al, 2000; Norat et al, 2002). In epidemiological studies where the effect of diet on colon cancer risk is examined, it is difficult to differentiate the effect of protein from other factors such as fat and calcium. Improvements in technology have enabled the production of protein concentrates and isolates (Smithers et al, 1996). These products can then be used to examine the effects of proteins (independent of other associated nutrients) on colon cancer risk, along with possible underlying mechanisms.

Animal studies have shown that whey protein, in comparison to red meat, soy bean meal and/or casein, is more protective against carcinogen-induced colon tumor expression (Bounous et al, 1988; Papenburg et al, 1990; McIntosh et al, 1995; Hakkak et al, 2001). Although these studies have suggested possible mechanisms for how whey protein may reduce colon cancer risk, the exact mechanism is still unknown. Whey protein has been shown to stimulate the immune system (Bounous et al, 1988; Bounous et al, 1989), to stimulate glutathione in liver or plasma (McIntosh et al, 1995; Lothian et al, 2000; Micke et al, 2001; Micke et al, 2002), to act as an antioxidant in-vitro (Colbert and Decker,

1991; Tong et al, 2000), to bind bile acids in-vitro (Kruidenier et al, 1985) and reduce fecal fat (McIntosh et al, 1995).

The effect of dietary protein concentration on colon cancer risk has not been clearly determined by epidemiological or animal studies. A study by Tatsuta et al (1992) investigated the effect of dietary protein concentration on azoxy-methane (AOM) induced colon tumors and showed that the administration of low (10%) or very low (5%) protein diets compared to the high protein diet (25%) significantly increased the incidence and number of colon tumors. This increase in colon carcinogenesis was correlated with an increase in nor-epinephrine concentration and cell proliferation in the colon mucosa. In contrast, Clinton et al (1992) did not show a significant influence of protein density on intestinal tumors and suggested that energy intake and dietary fat were the prime factors influencing tumor expression. Apart from these studies that used casein as the protein source, there is limited evidence as to the effect of increasing the density of other proteins on colon cancer risk.

Animal studies that have examined the effect of red meat on carcinogen induced ACF or tumors are inconsistent. In comparison to other protein sources (primarily casein), red meat has been shown to promote colon cancer risk in three studies (Goldin et al, 1980; Nutter et al, 1983; McIntosh et al, 1995) and to have no effect in five studies (Clinton et al, 1979; Pence et al, 1995; Lai et al, 1997; Parnaud et al, 1998). A protective effect was only found for red meat when a mouse model was used which contradicts the rat model data (Nutter et al, 1990). Further variation may have been caused by the use of different

sources and quantities of red meat, the cooking method used and/or varying quantities of other dietary components such as fat that may interact with red meat.

The heme iron component of red meat has been suggested as a factor contributing to increased colon cancer risk (Sesink et al, 1999). Heme iron in comparison to non-heme iron (ferric citrate) and metabolites of heme iron (bilirubin, protoporphyrin) has been shown to increase fecal water cytotoxicity and colon cell proliferation that are known risk markers for colon cancer. Furthermore, a high heme diet increased ACF and tumor expression in the colon of rats (Sawa et al, 1998; Pierre et al, 2003). However, in these studies heme was added to the diet at very high concentrations. It is not clear whether a diet high in red meat will have the same effect on risk markers for colon cancer as seen when heme iron was fed in previous studies.

The purpose of this study was to determine whether increasing the density of red meat or whey protein in the diet would alter the number of AOM-induced aberrant crypt foci (ACF) in rats. The degree to which diet altered the expression of aberrant crypt foci, preneoplastic lesions, was used as a biomarker of the development of colon cancer (Srivastava et al, 2001). Secondary objectives of the study were to determine whether 1) increased dietary heme iron in the red meat diets were directly related to increased heme iron and lipid peroxidation in fecal water, and 2) the quantity of dietary red meat or whey protein altered the amount of fat and protein that entered the colon.

5.2 Experimental design

5.2.1 Animals and diets

Seventy-four 13 week-old outbred Wistar rats were purchased from the Laboratory Animal Services Branch, Adelaide University. Animals were housed in wire cages to minimize coprophagy and maintained in an air-conditioned environment of 23 ± 2°C with a 12:12-h light-dark cycle. Upon arrival rats were separated by weight into six groups and started on their experimental diets. Rats were weighed weekly and average diet intake/cage of six rats was recorded at weeks 1, 4 and 8 whilst on the experimental diets. After four weeks on the experimental diets rats were s.c. injected once a week for two weeks with AOM (Sigma, St Louis, MO) dissolved in normal saline at a dose of 15mg/kg body weight. All experimental procedures using animals were approved by the Commonwealth Scientific and Industrial Research Organisation, Health Sciences and Nutrition Animal Ethics Committee and by the University of Adelaide Animal Ethics Committee.

The experimental diets were based on a modified AIN-93 purified diet with high fat (20% in total, by adding sunflower seed oil), low calcium (0.1%) and low fibre (2%) supplied as α-cellulose (**Table 5.1**). Protein quality and concentration varied between dietary treatment groups. To achieve total dietary protein concentrations of 8, 16 or 32%, protein was added as either barbecued kangaroo muscle meat or whey protein concentrate. The protein quantity of the diets was adjusted using carbohydrate and the ratio between sugar and starch was maintained in all diets. Minerals provided by the different protein sources were balanced in all diets. Ferric citrate was added to diets to obtain a concentration of 35 mg iron/kg.

Whey protein concentrate was supplied by NZ milk products (New Zealand) and contained 78% protein, 5.2% fat, 4.9% lactose and 4.2% moisture (**Table 2.2**). Kangaroo muscle meat was purchased from MacroMeat (Adelaide, South Australia). Prior to use, the meat was cooked on a barbecue with a hotplate temperature of 150°C (measured using an infrared thermometer, Rayranger, Raytek, California, USA). The meat was then dried at 45°C for 48 h and milled to provide a product containing 78% protein, 15.3% fat and 1% moisture (**Table 2.2**). The final meat product contained 5.2mg heme iron/100g and did not contain any meat mutagens (PhIP, MeIQ or IQ) (assayed by Mark Knize at the Lawrence Livermore National Laboratory, California, USA).

5.2.2 Sample collection and autopsy

In the final four weeks of the experiment rats were placed in metabolic cages to record and collect fecal output. At the conclusion of the experiment the rats were euthanased using 4% halothane/oxygen and blood was removed by abdominal exsanguination. The colon (cecum to anus) was removed and rinsed with saline, opened longitudinally, flattened onto blotting paper and fixed in 10% buffered formalin overnight (Tudek et al, 1989). The tissue was divided into two equal segments (proximal and distal) between the peyers patch and the herring bone and stained with 0.2% methylene blue in phosphate-buffered saline for 20 minutes. Aberrant crypts were distinguished from the surrounding normal crypts by fulfilling the following criteria: increased size, slit like lumen, thicker cell wall and raised crypt. Using a light microscope (50 x magnification), the number of aberrant crypts and foci were recorded for both the proximal and distal colon.

5.2.3 Analytical methods

Fecal water was prepared by the method of Lapre et al (1991) and Sesink et al (1999). In brief, freeze dried feces were reconstituted with milli-Q water based on a 30:70 ratio. Samples were vortexed vigorously, incubated for 1 h at 37°C and then centrifuged at 26,000 g. The supernatant was aspirated and osmolarity measured to ensure that it was 300 mOsM/L (Fiske Osmometer, Bethel Conn, USA). The amount of intact heme in the fecal water fraction of feces was measured by the Hemoquant assay (Schwartz et al, 1983) using hemin as the standard. Thiobarbituric acid reactive substance (TBARS) was measured in the fecal water following the method of Sesink et al (1999). 1,1,3,3-tetramethoxypropane was used as a standard and TBARS was reported as malondialdehyde equivalents.

Fecal fat was determined gravimetrically. Fat was extracted from the feces by an acidified chloroform:methanol extraction as described by Folch et al (1957). The weight of fat extracted was compared to the original weight of freeze dried feces. Fecal protein was quantified by the Dumas method (Kirsten and Hesselius, 1983) using a Carlo Erba NA 1500 nitrogen analyzer (Milan, Italy).

To determine fecal moisture, fecal samples from the rats were collected within 15 minutes of excretion, were weighed and then freeze dried. The difference between wet and dry weights was calculated.

5.2.4 Statistical analysis

Statistical analyses were performed using SPSS 10.0 for windows (SPSS Inc, Chicago, USA). The effect of dietary protein type and density on final body weight and weight gain was examined by between factor analysis of variance, with initial weight used as a co-variate. Group results were compared using a one-way analysis of variance test followed by a Tukey's multiple comparison test. Pooled values for protein type (whey protein, red meat) and density (8%, 16%, and 32%) were also compared using a one-way analysis of variance test followed by Tukey's multiple comparison test. Regression analysis was used to determine whether there was a relationship between the concentration of whey protein or red meat with fecal measures. Values were expressed as mean ± SE and differences between the treatment means were only considered significant when $p<0.05$.

5.3 Results

Diet intake was not significantly affected by either protein type or density (data not shown). The effect of protein type and density on growth rate and final body weight is shown in

Figure 5.1. Protein type and density significantly altered body weight gain (g/wk). An increase in the density of red meat in the diet was associated with an increase in weight gain ($p<0.05$). By contrast, increased density of whey protein in the diet was associated

inversely with weight gain ($p<0.01$). Final body weight of rats in the whey protein group was less than rats in the red meat group ($p<0.05$).

The effect of protein type and density on ACF number in the proximal and distal colon is summarized in **Figure 5.2**. In the proximal colon there was a 50% reduction of single ACF in the 32% whey protein group compared to the 16% and 32% red meat groups ($p<0.05$). In the distal colon protein density, independent of protein type, was associated with a reduction in the number of large ACF. Rats fed diets containing 8% protein had twice as many large ACF (5 or more) than those fed the 16% protein diets (4 ± 0.7 and 2 ± 0.5 ACF respectively, $p<0.05$).

The composition of the feces and fecal water is shown in **Table 5.2.** Mean daily fecal output was not different between dietary treatment groups (data not shown). The 8% red meat group had the highest concentration of fat in the feces (14%) relative to the other groups ($p<0.05$), but was not different from the 32% red meat group. The red meat fed rats overall had a higher percentage of fat in the feces than the whey protein fed rats (13 ± 0.2 vs 12 ± 0.2, $p<0.05$).

Protein type (whey protein or red meat) did not affect the quantity of protein that reached the colon (**Table 5.2**). However, increasing the density of protein in the diet was positively correlated to the quantity of protein (measured as nitrogen) in the feces ($r=0.83$, $p<0.001$).

Fecal moisture was affected by the type of protein in the diet (**Table 5.2**). Density of whey protein was positively correlated with fecal moisture ($r=0.44$, $p<0.05$). The density of red meat in the diet did not affect the amount of moisture in the feces.

The red meat diets contained heme iron that was not present in the other diets. To determine how much soluble intact-heme iron was present in the feces, the quantity of heme iron was measured in the fecal water component (**Table 5.2**). The concentration of dietary red meat was positively correlated with the concentration of heme iron in the fecal water ($r=0.62$, $p<0.001$). However, only the 32% red meat diet significantly increased the concentration of heme iron in the fecal water relative to the other dietary treatment groups.

TBARS was used to measure peroxidation of lipid in fecal water. It was shown that TBARS formation in the fecal water was positively related to the concentration of red meat in the diet ($r = 0.33$, $p<0.001$) (**Table 5.2**). Increasing the concentration of whey protein in the diet did not influence the concentration of TBARS in fecal water.

5.4 Discussion

Increasing the density of whey protein in the diet to 32% reduced the expression of single ACF in the proximal colon by approximately 50% in comparison to the red meat (16% and 32%) groups. Insofar as representing a reduced risk of tumorigenesis, these results are consistent with longer term studies that showed whey protein to reduce tumor incidence by about 45% and tumor burden by 60% relative to red meat (McIntosh et al,

1995; McIntosh et al, 1998). Larger ACF (four or more aberrant crypts per focus) have been considered more likely to progress to tumors (Zhang et al, 1992; Caderni et al, 1995) and in this study protein density, but not protein type, had a significant inverse influence on large ACF in the distal colon. A study by Tatsuta et al (1992) showed that the administration of low (10%) and very low (5%) protein diets compared to the normal protein diet (25%) significantly increased the incidence and number of AOM induced colon tumors. The increase in colon carcinogenesis correlated with an increase in nor-epinephrine concentration and cell proliferation in the colon epithelium of the animals consuming low and very low protein diets. However, Clinton et al (1992) failed to show a significant influence of protein concentration on intestinal tumors, and proposed that energy intake and dietary fat were the influential factors on tumor expression.

In this study the high whey protein diet had a profound influence on body weight gain of the rats, reducing it from about 14g/week/rat (32% red meat fed rats) to 8.7 (32% whey protein fed rats), despite there being no measurable effect on feed intake. The differences in weight gain may be due to altered plasma insulin, growth hormone and/or IGF-I and its binding proteins. These hormones that stimulate growth are widely believed to play a central role in carcinogenesis due to their important role in the regulation of cell cycle (Aaronson et al, 1990). Epidemiological studies have shown that individuals have an increased risk of colon cancer if they had type II diabetes, (Hu et al, 1999) a high basal secretion of IGF-I, a low plasma concentration of IGF-I binding protein (Ma et al, 1999) or if they have acromegaly, a condition characterized by chronic growth hormone and IGF-I (Klein et al, 1982; Ituarte et al, 1984). Animal studies support an association between growth factors and their promotion of colon cancer risk (Bruce et al, 2000).

Whey proteins on the other hand may protect against colon cancer risk by increasing liver glutathione concentration (McIntosh et al, 1995). Whey protein, containing a high concentration of cysteine (4.6 fold more than red meat) has been shown to stimulate the concentration of glutathione in liver of rats (McIntosh et al, 1995) and to significantly increase hepatic GST activities (Rowlands et al, 2000). An elevation in liver glutathione concentration may increase detoxification of free radicals produced by the metabolism of carcinogenic and xenobiotic compounds resulting in less of these agents reaching the colon to induce genetic damage.

Other components of the whey protein concentrate such as lactose, sphingolipids or conjugated linoleic acid may have provided some protection against chemical induced carcinogenesis. Recently, a prospective cohort study showed a strong inverse association between lactose intake and colon cancer risk (Jarvinen et al, 2001). Furthermore, dietary lactulose (0.3% of diet) has been shown to inhibit DMH-induced DNA damage to colonic crypt stem cells of human flora colonized rats (Rowland et al, 1996). In the current study there was a positive relationship between the density of whey protein in the diet and fecal moisture. Even though the lipid component of whey protein was low (approximately 8%), only small concentrations of conjugated linoleic acid (0.5%) and sphingolipids (0.025% of diet) are required to have a protective effect in the colon (Schmelz et al, 2000; Park et al, 2001).

High intakes of heme iron have been associated with increased risk of colon cancer either by inducing genetic damage to colonocytes or by stimulating the proliferation of the colonic epithelium. Sawa et al (1998) fed rats 1.76μM heme/g diet as hemoglobin and

found a two-fold increase in colon tumor expression. Free radical species are generated when the heme reacts with polyunsaturated fat in the colon and this reaction, in vitro has been shown to damage DNA (Akaike et al, 1992; Sawa et al, 1998). Heme when fed to animals at a similar concentration, 1.3μM/g as hemin, significantly increased fecal water cytotoxicty and proliferation rate of colon epithelium (Sesink et al, 1999). Furthermore, Sesink (2000) examined the dose response of feeding heme iron and showed that changes in fecal water cytotoxicity and proliferation were only seen when the concentration of heme in the diet was greater than 0.16μM/g diet and when the concentration of heme in the fecal water was greater than 42μM. The kangaroo red meat used in this study contained approximately twice as much heme iron as beef, and although cooked it did not contain any meat mutagens. In the current study, 32% red meat diet contained 0.38μM heme/g diet which was two fold higher than the heme diet which had previously been shown to have an effect. However, the concentration of heme in the fecal water was only 25.1μM. A reason why the 32% red meat diet did not increase colon cancer risk may be due to a difference in absorption properties of heme iron, which depends on the dietary form in which it is administered. Less heme iron reaches the colon when it is supplied as red meat rather than in the purified form. Only 25% of heme iron from red meat has been found to reach the colon, whereas 97% of hemin and 88% of hemoglobin passed into the colon (Schwartz and Ellefson, 1985; Sesink et al, 1999). Although there was an increase in lipid peroxidation in fecal water in the current study (as measured by TBARS) this was approximately half that seen in a previous study where a cytotoxic effect of heme was seen (Sesink et al, 1999). These findings suggest that heme iron rich red meat diets are not sufficient to increase the concentration of heme iron in the fecal water to a level

which has been shown to stimulate tumor growth or increase fecal water cytotoxicity and proliferation in the colon.

In summary, using ACF number as a relevant measure, increasing the density of WPC in the diet to 32% was more protective in comparison to red meat (16% and 32%) which supports earlier tumorigenesis studies. The lack of a promotional effect with increasing red meat may be due to low amounts of heme reaching the colon, to induce a cytotoxic effect. Factors such as body weight gain or differing levels of fat in feces may explain in part the altered number of ACF in this study.

Table 5.1. Composition of experimental diets based on AIN-93 formulation[1]

	Red meat			Whey protein		
	8%	16%	32%	8%	16%	32%
Red meat[2]	10.25	20.5	41			
Whey protein[3]				10.25	20.5	41
Sugar	25.86	22.36	15.44	25.56	21.8	14.28
Cornstarch	38.79	33.54	23.16	38.38	32.7	21.42
Fibre	2	2	2	2	2	2
Oil[4]	18.4	16.9	13.7	19.15	18.3	16.6
Choline	0.2	0.2	0.2	0.2	0.2	0.2
Minerals	3.5	3.5	3.5	3.5	3.5	3.5
Vitamins	1	1	1	1	1	1

[1]Values are expressed as g/100g
[2] Red meat is a barbecued kangaroo muscle meat. It contains 78% protein, 15.3% fat and 1% moisture
[3] Whey protein contains 78% protein, 8.3% fat, 4.9% lactose and 4.2% moisture
[4]Amounts of Sunflower seed oil was added to diet to provide 20% fat (w/w). Protein sources provided the remainder.

Table 5.2. Fecal composition data [1,2]

		Fecal Fat (% dry wt)	Fecal Protein (% dry wt)	Fecal Moisture (%)	Heme in fecal water (μM)	TBARS in fecal water (μM MDA equivalents)
Red meat	8%	14 ± 0.5a	16 ± 0.6c	44 ± 2.1b	1.1 ± 0.2b	39 ± 4.8bc
	16%	12 ± 0.2b	17 ± 0.6ab	54 ± 1.4ab	4.2 ± 0.9b	58 ± 6.5ab
	32%	12 ± 0.3ab	22 ± 0.9a	48 ± 3.9ab	25.1 ± 6.0a	79 ± 8.2a
Correlation		ns	0.001	ns	0.001	0.001
Whey protein	8%	12 ± 0.2b	15 ± 0.7c	50 ± 3.0ab	0.6 ± 0.2b	30 ± 4.6c
	16%	12 ± 0.1b	18 ± 0.7b	49 ± 4.6ab	0.9 ± 0.3b	33 ± 4.5c
	32%	11 ± 0.3b	22 ± 0.4a	60 ± 2.9a	1.4 ± 0.6b	27 ± 2.6c
Correlation		ns	0.001	0.03	ns	ns
ANOVA[3]						
Protein type		0.009	ns	ns	0.001	0.001
Protein density		0.009	0.001	ns	0.001	0.006
Type*density		0.028	ns	ns	0.001	0.001

[1] Data is presented as mean ± SE

[2] Different superscript letter denotes significant difference between dietary treatment groups down columns ($P<0.05$)

[3] p-value shown if < 0.05

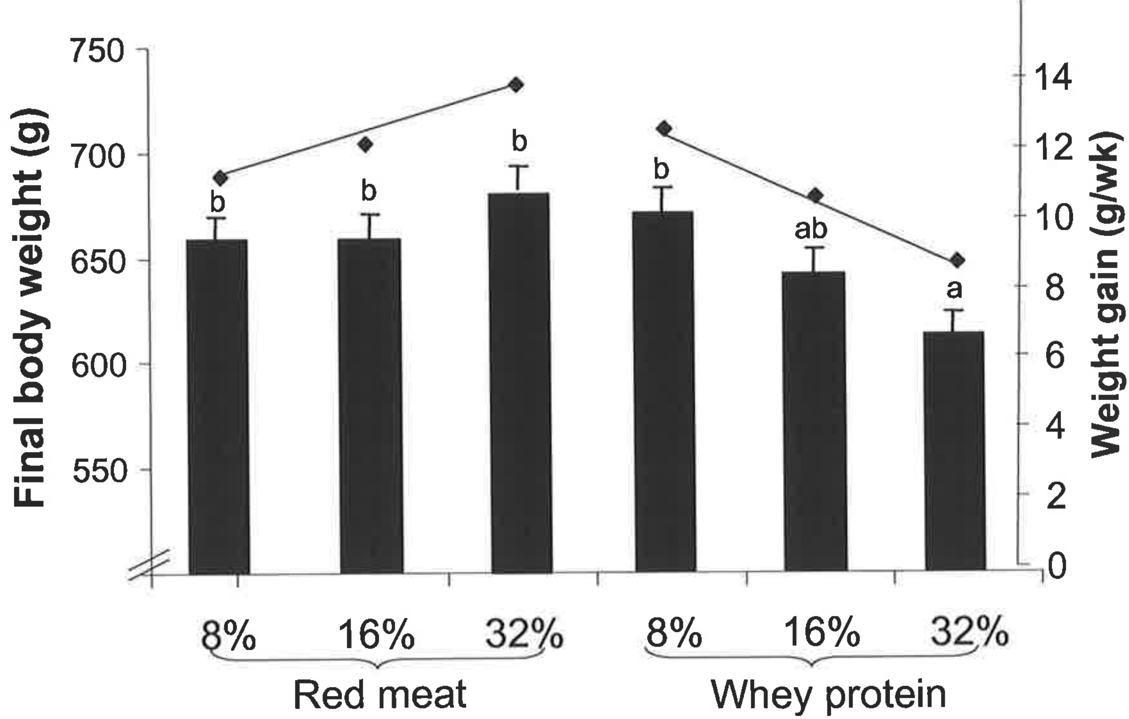
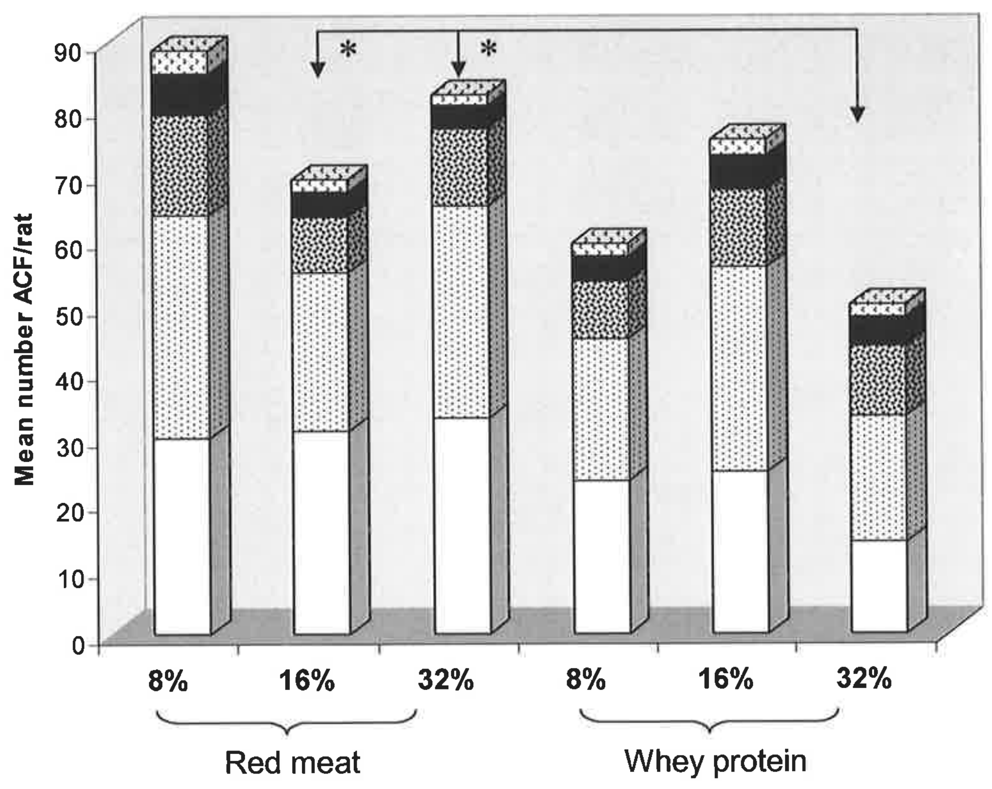
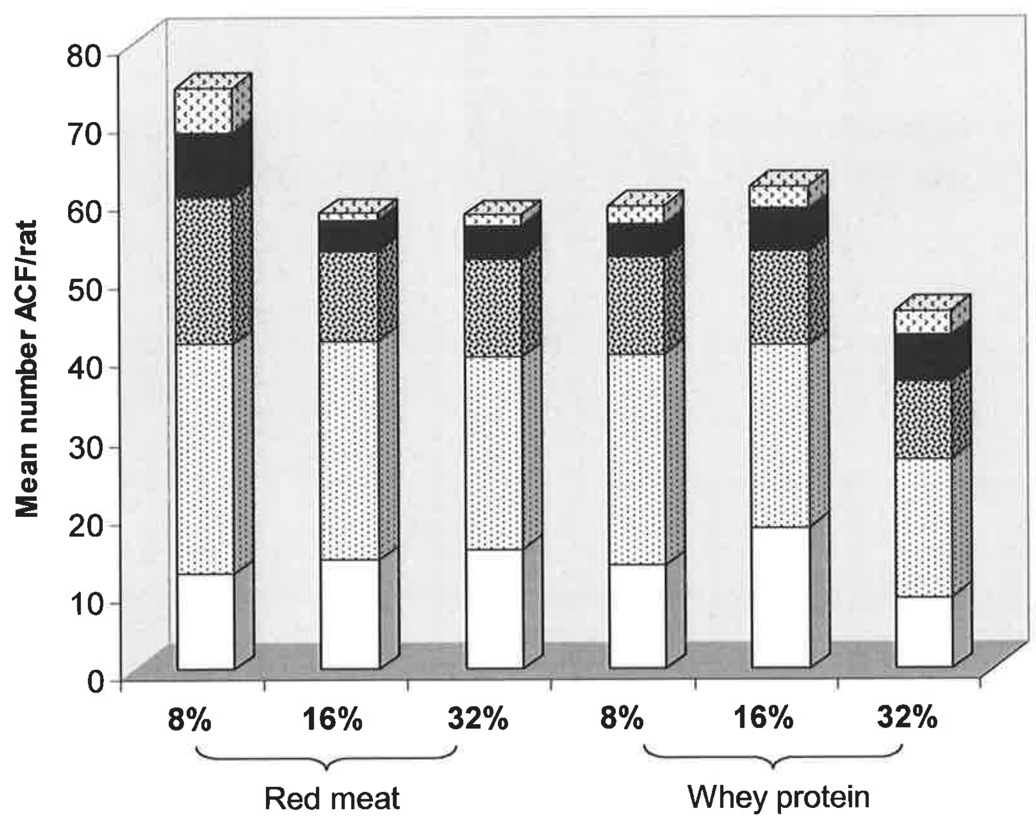


Figure 5.1. The effect of protein type and density on final body weight and weight gain.

Final body weight is depicted in columns, different letter denotes significant difference between dietary treatment groups ($p<0.05$). Weight gain for dietary treatment groups is shown on the right axis (◆). The lines depict the changes in weight gain for increasing density of protein, as red meat ($p<0.05$) or whey protein ($p<0.01$).



a.



b.

Figure 5.2. The effect of protein type and density on expression of ACF in the proximal colon (a) and distal colon (b). Number of crypts per focus are 1: □ , 2: ▫ , 3: ▩ , 4: ■ , 5: ⬚ A (*) denotes significantly less single ACFs in the 32% whey protein group compared to the 16% and 32% red meat groups ($p<0.05$).

Chapter 6 – HIGH PROTEIN DIET OF WHEY PROTEIN REDUCES BODY WEIGHT GAIN AND IMPROVES INSULIN SENSITIVITY RELATIVE TO RED MEAT IN THE WISTAR RAT

6.1 Introduction

The prevalence of non-insulin dependent diabetes mellitus (NIDDM) is increasing rapidly in the United States (Flegal et al, 1998; Mokdad et al, 2000) and worldwide (King et al, 1998). Obesity is commonly associated with insulin resistance, which is considered one of the earliest detectable and major predisposing conditions in people with NIDDM (Lillioja et al, 1988; Eriksson et al, 1989). Insulin resistance is characterized by impaired insulin-stimulated glucose disposal by skeletal muscle (Roberts et al, 2000). Although genetic predisposition is a significant factor in insulin resistance, diet and lifestyle factors may also play a key role in the development of this condition. The Western diet, characterized by elevated intakes of red meat, saturated fat and refined carbohydrates and low intakes of fibre and calcium has been associated with an increased risk of insulin resistance and obesity (van Dam et al, 2002).

At present, energy restriction is the most effective way for people with insulin resistance, obesity and/or NIDDM to improve glucose control and plasma lipid profile and lose weight (Henry et al, 1985; Kelley et al, 1993). Weight loss by this method has been shown to alter the levels of circulating hormones associated with the regulation of satiety (ghrelin, glucagon-like-peptide-1 and leptin) (Considine et al, 1996; Havel et al, 1996; Verdich et al, 2001; Cummings et al, 2002). This reduces satiety and increases appetite, which makes it difficult to adhere to an energy restricted diet. A more effective way to improve insulin sensitivity, fat and weight loss may be through altering the macronutrient composition of the diet. Increasing the density of protein in the diet has been shown to reduce energy intake by increasing satiety (Crovetti et al, 1998) and to increase total

energy expenditure by the increased thermogenesis associated with protein digestion (Crovetti et al, 1998; Johnston et al, 2002). A high protein diet fed ad libitum reduced energy intake and increased weight and fat loss in obese subjects in comparison to a low protein diet (Skov et al, 1999). When energy intake was restricted, only one study showed that a high protein diet reduced abdominal fat and/or body weight (Baba et al, 1999) and nine studies showed no effect compared with a similarly energy restricted low protein diet (DeHaven et al, 1980; Yang and Van Itallie, 1984; Alford et al, 1990; Piatti et al, 1994; Vazquez et al, 1995; Luscombe et al, 2002; Parker et al, 2002; Farnsworth et al, 2003; Moran et al, 2003). As a mixed protein meal was used in these studies, it is not clear whether protein type (red meat, dairy, vegetable protein) may have a significant impact on the effectiveness of a high protein diet in reducing body weight and fat storage and in improving insulin sensitivity.

Animal studies have shown that red meat increased body weight in comparison to dairy protein (casein, whey protein) (McIntosh et al, 1995; Lai et al, 1997). Increasing the density of red meat in the diet was also positively associated with weight gain in rats (Parnaud et al, 1998; Belobrajdic et al, 2003). Alternatively, whey protein has been shown to reduce body weight in rats when fed at moderate (Hakkak et al, 2000; Minehira et al, 2000; Badger et al, 2001) and at high levels (Chapter 5). As these studies have been conducted in predominately young growing animals, it is not known whether the protein diets would have similar effects on body weight in mature rats with insulin resistance. The outbred Wistar rat can be made insulin resistant by feeding it a diet high in fat (30% w/w) for at least three weeks (Storlien et al, 1986). Insulin resistance can be identified in these animals by increased visceral fat and muscle triglyceride accumulation, whole body

and skeletal muscle insulin resistance and hyperinsulinemia (Grundleger and Thenen, 1982; Kraegen et al, 1986; Storlien et al, 1991; Han et al, 1997; Kim et al, 2000). It was hypothesized that in insulin resistant Wistar rats, a high protein diet (32%) provided by whey protein will reduce weight gain and tissue lipids and improve insulin sensitivity to a greater extent than one containing red meat as the protein source.

6.2 Experimental design

6.2.1 Animals and diets

Male Wistar rats 10 weeks of age weighing 367 ± 6g (n=28) were obtained from the Laboratory Animal Services Branch, Adelaide University. Animals were housed in wire cages to minimize coprophagy and were maintained in an air-conditioned environment of 23 ± 2°C with a 12-h light/12-h dark cycle.

The rats were fed a high fat (300g fat/kg) AIN-93 based diet for 9 weeks. To ensure that rats were insulin resistant after 9 weeks on the high fat diet, blood was taken from the tail vein of overnight fasted rats (14 h) and insulin concentration was measured. Fasting insulin concentration in the current study (19.5 ± 3.1μU/ml) was similar to animals fed a high fat diet in a previous study (17.2 ± 2.1μU/ml) (Kim et al, 2000). These insulin concentrations were 2 fold higher than lab chow fed animals (Kim et al, 2000). The rats were then separated randomly into four dietary treatment groups. The diets contained either 8 or 32% protein provided by either whey protein concentrate whey protein or red meat. Diet intake was calculated daily for each rat as they were housed individually.

At the conclusion of the study the rats were fasted over night and euthanased using 4% fluothane/oxygen anesthesia. Blood was removed by exsanguination from the abdominal aorta. The visceral fat (mesenteric, epididymal and retroperitoneal) and subcutaneous fat pads were removed and weighed. The remaining internal organs, skin, feet, head and tail were then removed, leaving only the carcass (muscle mass and skeleton). All experimental procedures using animals were approved by the Commonwealth Scientific and Industrial Research Organisation, Health Sciences and Nutrition Animal Ethics Committee and the University of Adelaide Animal Ethics Committee.

The high fat diet contained 300g of fat (1:1 mixture of sunflower seed oil and beef fat), 510g sugar, 120g casein and 20g fibre as α-cellulose. Choline, cysteine, minerals and vitamins were added as outlined in the AIN-93 diet (Reeves et al, 1993). The experimental protein diets varied in protein type and density between treatment groups and fat was constant at 200g/kg (provided by a 1:1 mixture of sunflower seed oil and beef fat) (**Table 6.1**). To achieve total dietary protein concentrations of 8% or 32%, protein was added either as barbequed kangaroo red muscle meat, or whey protein. The protein density of the diets was adjusted using carbohydrate with a 3:2 ratio of starch to sugar. Minerals provided by the different protein types were balanced in all diets to a constant level. Calcium (1g/kg) and fibre (20g/kg as α-cellulose) were added to all diets at a low concentration to simulate the Western diet.

Whey protein concentrate contained 78% protein, 5.2% fat, 4.9% lactose and 4.2% moisture and was supplied by NZ milk products (New Zealand). Red meat was kangaroo muscle (red) meat purchased from MacroMeat (Adelaide, South Australia). The meat was

cooked on a barbeque (150°C), dried at 45°C for 48 h and milled to provide a stable product for diet preparation. The final product contained 78% protein, 15.3% fat and 1% moisture.

6.2.2 Carcass analysis

Each animal carcass was freeze dried, minced and then ground using a hammer mill to provide a fine powder for analysis. Carcass fat was determined gravimetrically, following extraction with 2:1:1 chloroform:methanol:water as described by Folch et al (1957). The weight of fat extracted was expressed as g/100g of tissue. Carcass protein was quantified by the Dumas method (Kirsten and Hesselius, 1983) using a Carlo Erba NA 1500 nitrogen analyser (Milan, Italy). The quantity of bone in the carcass was based on calcium values. Carcass samples were acid digested and then analysed by atomic absorption spectrometry for calcium (Varian, Victoria, Australia).

6.2.3 Blood chemistry and hormone analysis

The plasma insulin concentration was determined by radioimmunoassay (Linco Research, Rat insulin RIA, Missouri, USA) at 9 weeks and by ELISA (ALPCO, Rat insulin EIA, New Hampshire, USA) at the end of the study. Plasma glucose and triglycerides were determined spectrophotometrically using commercially available kits (Gluco-quant®, Roche, Indianapolis, USA, Triglycerides GPO-PAP, Roche, Indianapolis, USA). Plasma free fatty acid was measured by an enzymatic fluorometric method (Roche, Mannheim, Germany). Plasma IGF-I was measured by ELISA (Immunodiagnostic Systems, Boldon, UK). Thyroid hormones, T3 and T4 were measured by radioimmunoassay (Brahms, Hennigsdorf, Germany). Insulin resistance was quantified in the rats using the insulin to

glucose ratio which has been shown to correlate strongly with measures obtained from the euglycemic-hyperinsulinemic clamp technique (Ahren and Larsson, 2002).

6.2.4 Statistics

Statistical analyses were performed using SPSS 10.0 for windows (SPSS Inc, Chicago, USA). The effects of protein type and density on parameters were determined by a two-way analysis of variance test followed by Tukey's multiple comparison test. The effect of dietary protein type and density on body weight (weeks 10-17), was examined by between factor and repeated measures analysis of variance, with weight at week nine as a co-variate. The effect of dietary protein type and density on dietary intake was examined using between factor and repeated measures factor analysis of variance. Values were expressed as mean and standard error of the mean and differences between the treatment means were considered significant when $p<0.05$.

6.3 Results

6.3.1 Weight gain and food intake

Energy intake was inversely related to protein density of diet, such that animals in the low protein groups consumed 19% more energy per day compared to animals in the high protein groups ($p<0.05$) (Figure 6.1). However, protein type did not affect energy intake. Following the high fat preconditioning period, the 32% whey protein fed animals gained less weight than the other protein groups (4% less weight than 8% whey protein and 8% red meat groups and 10% less weight than 32% red meat group, $p<0.001$).

6.3.2 Body composition and tissue lipids

The effect of protein density and type on visceral and subcutaneous fat depots is shown in Table 6.2. The amount of visceral and subcutaneous fat was inversely related to protein density ($p<0.001$). Increasing the density of protein from 8 to 32% decreased visceral fat and subcutaneous fat weight by 22.6% and 25.6% respectively. Visceral fat was less in the 32% whey protein fed animals in comparison to 8% red meat and 8% whey protein fed animals ($p<0.05$). Subcutaneous fat was significantly less in the 32% whey protein fed animals in comparison to the 8% red meat fed animals ($p<0.05$). The fat pads of the 32% red meat fed animals were not different from any of the other dietary treatment groups.

The composition of the carcass was affected by the type and density of protein in the diets (Table 6.2). Increased protein density in the diet increased carcass protein by 9.4% ($p<0.05$) and decreased fat quantity in the carcass by 8.6% ($p<0.05$). The carcass of the 32% whey protein fed animals had less fat and more skeletal muscle than the 8% red meat fed animals, but was not different from the 32% red meat fed animals (Table 6.2). There was a trend to a reduction in bone density for animals fed whey protein in comparison to red meat ($p=0.05$).

6.3.3 Circulating lipids and insulin sensitivity

WPC fed animals had reduced plasma insulin concentration in comparison to red meat fed animals ($p<0.05$) (**Table 6.3**). Although plasma glucose concentration was not different, the insulin/glucose ratio, a measure of insulin resistance was lower for the whey protein fed animals in comparison to the red meat fed animals ($p<0.05$). Protein density

lowered plasma triglyceride and IGF-I concentration ($p<0.05$) but did not affect plasma glucose, insulin or free fatty acid concentration (**Table 6.3**). Protein type and density did not effect the concentration of thyroid hormones (T3 and T4) or corticosterone in the plasma (data not shown).

6.4 Discussion

The present study has shown that a high protein diet, irrespective of protein type was effective in reducing the storage of visceral, subcutaneous and carcass fat in the mature rat by up to 26%. This reduction in body fat following a high protein diet can be partially explained by a 19% decrease in energy intake. This finding is in agreement with previous studies in the rat which have shown that on a weight for weight basis, protein is more satiating than carbohydrate and fat (Jean et al, 2001; Bensaid et al, 2003). Thus animals on a high protein diet consume less energy which may otherwise be stored as fat. The reduction in fat storage for animals consuming the high protein diet may also be due to the metabolic cost of digesting protein. Energy expenditure has been shown to increase by up to 160% following a high protein meal in comparison to a high carbohydrate or fat meal (Crovetti et al, 1998). Although the thermic effect of food is only a small proportion of daily energy expenditure, the change seen in the study by Crovetti et al (1998) in relation to the high protein diet over 3 meals could equate to a considerable increase (8.4% or 480kJ) in energy expenditure per day. The high protein diet fed animals also had a reduced fasting plasma triglyceride concentration, which suggests that there is a reduction in VLDL production by the liver. This reduction in plasma triglyceride has been shown previously with high protein diets, where a reduction in body weight or visceral fat was also seen in human subjects (Baba et al, 1999; Skov et al, 1999; Samaha

et al, 2003). Another beneficial effect of the high protein diet was a sparing of lean body mass, whereby total muscle protein of the carcass increased and total carcass fat decreased. These changes in body composition which are predominantly caused by a reduction in energy intake, do not account for the reduction in IGF-I concentration in the plasma. Previous studies have shown that increasing the protein level in the diet did not effect circulating IGF-I concentrations, which was only lowered in rats fed very low protein diets (Dardevet et al, 1991; Hirschberg and Kopple, 1991; Filho et al, 1999; Noguchi, 2000). The reason for this reduction in IGF-I on the high protein diet in the current study is not clear and requires further investigation.

In the scientific literature there is consistent evidence that fasting plasma insulin in glucose homeostasis regulation is affected by changes in visceral fat mass (Lemieux et al, 1996). The current study showed that increasing protein density in the diet reduced fat storage, but did not affect fasted plasma insulin, glucose or the insulin/glucose ratio. This may be explained by the differing actions of whey protein and red meat on metabolism.

There is increasing evidence that the inclusion of whey protein in the diet reduces weight gain (Hakkak et al, 2000; Minehira et al, 2000; Badger et al, 2001). The current study showed that increasing the density of protein in the diet was only effective in reducing weight gain when whey protein was the protein source, not red meat. This supports our previous study, that showed increasing the density of whey protein was inversely associated with weight gain and final body weight (Chapter 5). Other studies that have examined the effect of whey protein at moderate intakes in growing rats have shown a

reduction in growth rate and final body weight in comparison to casein (Hakkak et al, 2000; Minehira et al, 2000; Badger et al, 2001).

In the current study, the 32% whey protein fed animals showed an improvement in insulin sensitivity that was reflected by a reduction in the fasting plasma insulin concentration. These changes may be accounted by the reductions seen in visceral fat for the 32% whey protein fed rats compared to the low protein fed rats, as visceral obesity correlates strongly with insulin resistance (Lemieux et al, 1996; Yamashita et al, 1996; Bonora, 2000). These findings also suggest that visceral fat is mobilized and/or utilized as a result of whey protein intake. The high whey protein fed rats also had a lower fasting triglyceride concentration which indicates that there is less VLDL produced by the liver.

It was postulated that whey protein consumption may reduce body fat storage and weight gain by stimulating the release and activity of hormones which increase metabolic rate. Thyroid hormones (T3 and T4) have been previously shown to increase in subjects consuming a high protein diet in comparison to a high carbohydrate diet (Layman et al, 2003). Also, a diet containing an high amount of tryptophan has been shown to increase plasma cortisol and ACTH concentration (Modlinger et al, 1979; Modlinger et al, 1980). The 32% whey protein fed animals consumed twice as much tryptophan as the 32% red meat fed animals (200mg and 100mg of tryptophan/day respectively). However, protein type and density did not affect fasting plasma corticosterone concentration or the concentration of thyroid hormones. As these hormones fluctuate in response to circadian rhythm, diet and activity, a more comprehensive examination of these hormones is required. Further understanding of the effects of high protein diets on metabolism may be

gained by examining the changes in these hormones over a 24 h period or following a meal.

Animal studies have shown that red meat increased body weight in comparison to whey protein (McIntosh et al, 1995; Lai et al, 1997). Increasing the density of red meat in the diet was also associated with weight gain in rats (Chapter 5) (Parnaud et al, 1998). In the current study older rats with insulin resistance were used. Rats consuming the high red meat diets grew at the same rate as low red meat fed animals. As the high red meat fed animals consumed significantly less energy than low red meat fed animals it was not clear why they grew at the same rate. Epidemiological studies show some evidence for a positive relationship between red meat dietary patterns and body mass index (Maskarinec et al, 2000; Newby et al, 2003). Maskarinec and colleages (2000) showed that a meat pattern diet (high in processed and red meats, fish, poultry, eggs, fats and oils, and condiments) was positively associated with BMI, independent of energy intake. In a prospective study by Newby et al (2003) using cluster analysis, the red meat and potato diet cluster was related to a greater increase in BMI over time compared to the control diet. Although the effect of red meat could not be separated from potato intake in that study it does suggest that the red meat diet may have an adverse effect on BMI.

Recently, there has been considerable interest in the use of high protein diets to improve weight loss and insulin sensitivity in people with insulin resistance, NIDDM and obesity. The current study suggests that the high protein diets reduced energy intake, lowered fat storage and increased the amount of skeletal muscle protein. Furthermore, in a high protein diet, protein type has differing effects on body weight, fat storage and insulin

sensitivity. Increasing the density of whey protein in the diet, but not red meat, reduced weight gain and improved insulin sensitivity by reducing fasting plasma insulin concentration. In conclusion, whey protein in a high protein diet may improve weight loss and insulin sensitivity to a greater extent than red meat.

Table 6.1. Composition of protein diets based on AIN-93 formulation[1]

	Red meat		Whey protein	
	8%	32%	8%	32%
Protein				
Red meat	104	418		
Whey protein			101	402
Sugar	258	151	255	141
Cornstarch	386	226	382	211
Fibre	20	20	20	20
Fat[2]	185	139	195	179
Choline	2	2	2	2
Minerals	35	35	35	35
Vitamins	10	10	10	10

[1] Values are expressed as g/kg

[2] Fat was added to diets as sunflower seed oil and beef fat (1:1) to achieve a total fat content of 200g/kg. Protein sources provided the remainder.

Table 6.2. Effect of diet on fat pad weight and carcass composition

	Red meat		Whey protein		ANOVA	
	8%	32%	8%	32%	Type	Density
Visceral fat pads						
Epididymal	2.7 ± 0.1 [a]	2.3 ± 0.1 [bc]	2.5 ± 0.1 [ab]	2.0 ± 0.1 [c]	0.033	0.001
Mesenteric	2.4 ± 0.2 [a]	1.9 ± 0.2 [ab]	2.1 ± 0.2 [ab]	1.6 ± 0.2 [b]	ns	0.021
Perinephric	4.3 ± 0.3 [a]	3.5 ± 0.3 [ab]	4.5 ± 0.3 [a]	3.0 ± 0.3 [b]	ns	0.001
Total	9.5 ± 0.4 [a]	7.7 ± 0.4 [bc]	9.0 ± 0.5 [ab]	6.6 ± 0.4 [c]	ns	0.001
Subcutaneous fat	9.7 ± 1.0 [a]	7.3 ± 0.7 [ab]	8.3 ± 0.8 [ab]	6.1 ± 0.6 [b]	ns	0.01
Mean Carcass wt, dry (g)	92.8 ± 3.8	94.3 ± 2.9	92.0 ± 2.5	86.9 ± 1.3	ns	ns
Carcass composition (%)						
Fat	23.7 ± 1.3 [a]	21.5 ± 1.2 [ab]	23.2 ± 1.4 [ab]	18.5 ± 1.3 [b]	ns	0.018
Protein	57.4 ± 1.6 [a]	59.6 ± 1.5 [ab]	59.3 ± 1.7 [ab]	63.9 ± 1.6 [b]	ns	0.049
Bone	3.8 ± 0.3	3.9 ± 0.3	3.2 ± 0.4	3.2 ± 0.3	ns	ns

Values are expressed as a percentage of final body weight. Different superscript letter in each row denotes significant difference between the dietary treatments ($p<0.05$)

Table 6.3. Effect of protein diets on fasted plasma metabolite and hormone concentration

	Red meat		**Whey protein**		**ANOVA**	
	8%	**32%**	**8%**	**32%**	**Type**	**Density**
Insulin (uU/ml)	29 ± 6	31 ± 5	24 ± 6	13 ± 5	0.03	ns
Glucose (mM/L)	6.9 ± 0.5	7.4 ± 0.4	7.3 ± 0.5	7.3 ± 0.4	ns	ns
Free fatty acid (uM/L)	609 ± 131	566 ± 111	786 ± 131	583 ± 120	ns	ns
Triglyceride (mM/L)	1.0 ± 0.2	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.1	ns	0.034
Insulin/glucose	4.3 ± 0.9 [a]	4.2 ± 0.9 [a]	3.3 ± 0.9 [ab]	1.7 ± 0.9 [b]	0.019	ns
IGF-I (ug/ml)	1.8 ± 0.1	1.7 ± 0.1	2.0 ± 0.1	1.7 ± .01	ns	0.024

Different superscript letter in each row denotes significant difference between the dietary treatments ($p<0.05$), n=6.

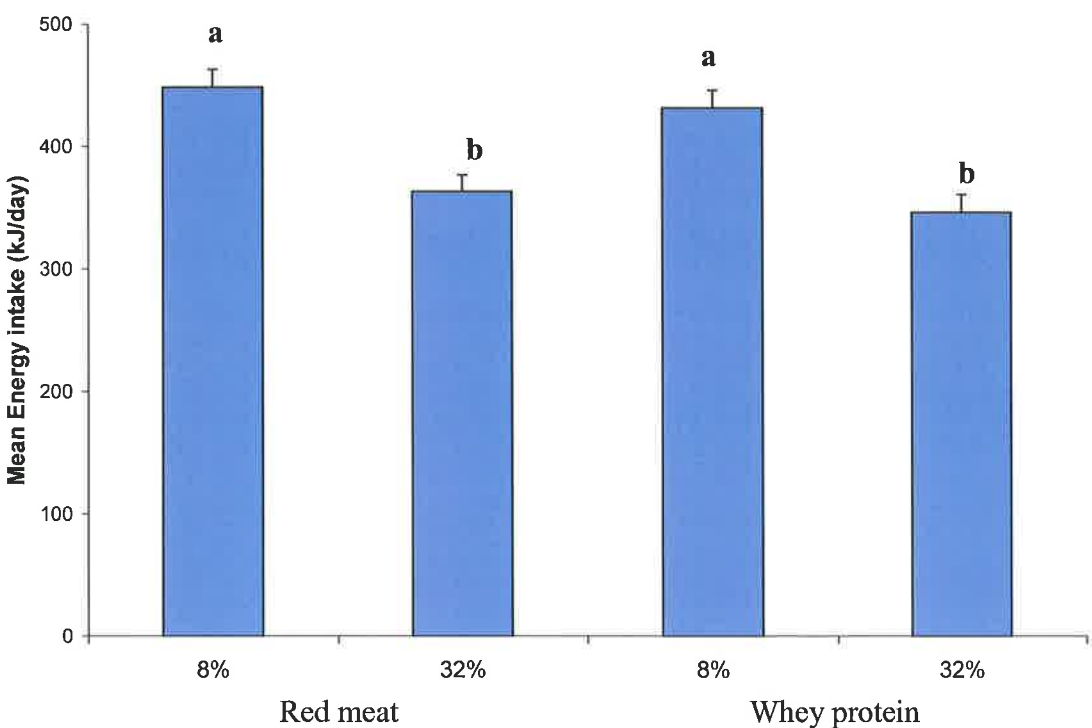


Figure 6.1. Effect of protein diets on daily energy intake. Different letter denotes significant difference between dietary treatment groups (p<0.05).

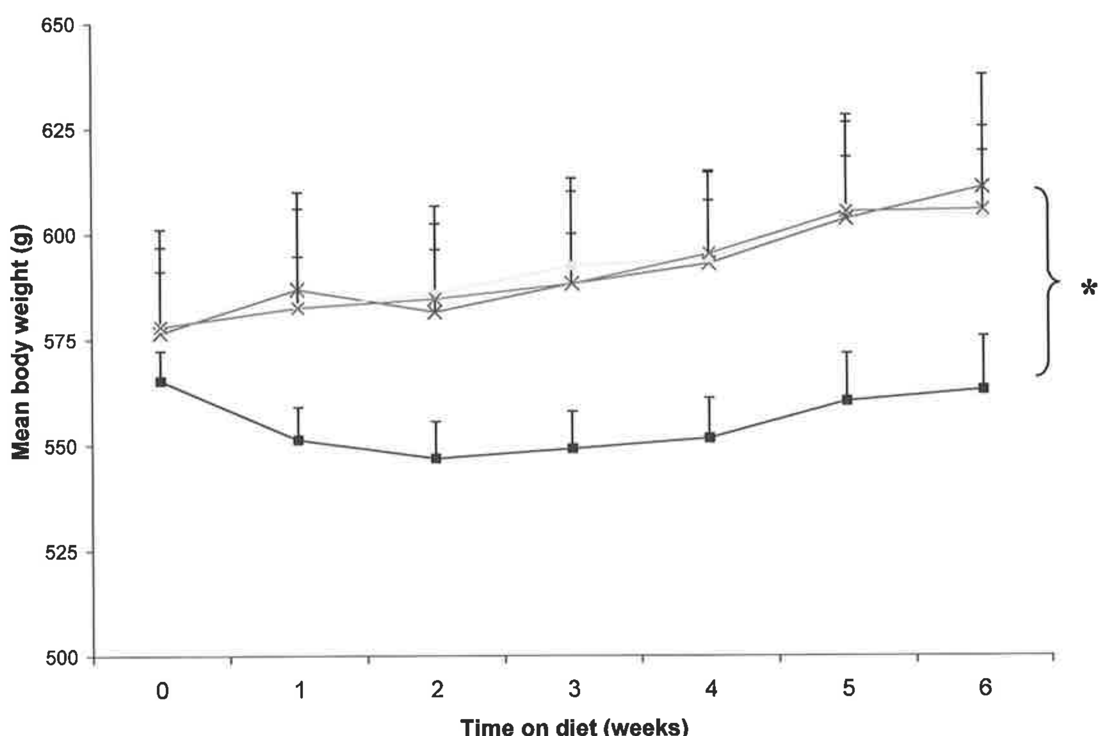


Figure 6.2. Effect of experimental diets on the body weight of rats. Red cross, 8% red meat; yellow square, 32% red meat; light blue cross, 8% whey protein; dark blue square, 32% whey protein. * denotes significant difference ($p < 0.001$).

Chapter 7 – GENERAL DISCUSSION

7.1 Red meat and colon cancer risk

7.1.1 Influence of fat type on the toxic effects of heme iron in the colon

Animal feeding studies have demonstrated that the addition of heme iron to a Western diet increased risk markers for colon cancer including, increased proliferation, lipid peroxidation, ACF and tumor number (Sawa et al, 1998; Sesink et al, 1999; Sesink et al, 2001; Pierre et al, 2003). Other dietary components may influence the damaging effects of heme in the colon. Sesink (2000) found that dietary calcium inhibited the cytotoxic and proliferative effects of heme in the colon, whereas fat quantity had no effect (Sesink et al, 2000). Fat type may play a significant role in affecting the toxic effects of heme in the colon. Substituting a polyunsaturated fat (sunflower seed oil) with either olive oil (Pierre et al, 2003) or milk fat (Chapter 3) reduced the toxic effect of heme iron in the colon. In high heme diets, olive oil in comparison to sunflower seed oil normalised the number of ACF, ACF size, fecal TBARS and fecal water cytotoxicity (Pierre et al, 2003). As described in Chapter 3, in high heme iron diets, AMF in comparison to sunflower seed oil did not increase fecal cations, fat and fecal water cytotoxicity. These findings suggest that a polyunsaturated fat such as sunflower seed oil must be present in the diet for heme to exert a toxic effect in the colon. This is consistent with an *in vitro* study by Sawa et al (1998) in which heme was incubated with different fat types. Only oxidized refined vegetable oils, such as safflower oil, readily generated lipid peroxyl radicals whereas the unpurified native vegetable oils did not. These lipid peroxyl radicals generated specifically from a heme, polyunsaturated fat mixture have been shown to induce cytotoxic and genotoxic damage and may explain why polyunsaturated fat, but not

saturated (AMF) or monounsaturated fat (olive oil) increased colon cancer risk in a high heme diet.

7.1.2 Heme iron: a possible initiator of colon cancer

For a dietary agent to be considered as an initiator of colon cancer it must be able to induce multiple genetic mutations in specific genes that regulate mucosal proliferation. Furthermore, these mutated cells must not be removed by apoptosis. There is now increasing evidence that supports the hypothesis that heme may initiate colon cancer. Firstly, heme iron has been shown to induce genetic damage to cells *in vitro* and *in vivo*. *In vitro* studies have shown that a mixture of heme iron and polyunsaturated fat induced cytotoxic effects to bacterial cells (Akaike et al, 1992; Akaike et al, 1995) and genotoxic damage to calf thymus and plasmid DNA (Sawa et al, 1998; Kanazawa et al, 2000). Furthermore, this genotoxic effect has been shown to occur to cells in the colon (Chapter 4). As described in Chapter 4, the addition of heme iron to a polyunsaturated fat based diet increased the amount of genetic damage in the colonocytes of rats. Secondly, heme iron has been shown to alter the expression of genes related to colon cancer. Pierre et al (2003) showed that amongst the most modulated genes, 18 out of 50 up regulated genes and 21 out of 30 down regulated genes were related to carcinogenesis. More specifically heme down-regulated the expression of the pentraxin gene, a gene involved in cell death, in the mucosa of the colon by more than 10 fold (Van der Meer Van Kraaij et al, 2003). Thirdly, heme iron has been shown to increase proliferation rate of epithelial cells in the colon (Sesink et al, 1999; Sesink, 2000). This increase in proliferation may increase the risk of a mistake occurring during DNA replication, which if not detected and removed by apoptosis, could initiate carcinogenesis. Finally, heme iron has been shown to act as

an initiator and/or promoter of colon cancer in animals injected with a carcinogen, by increasing the expression of ACF and tumors (Sawa et al, 1998; Pierre et al, 2003). However, in these studies it is not possible to identify whether heme was acting at the initiation or promotional stage of carcinogenesis.

7.1.3 Do high red meat diets contain sufficient heme to increase colon cancer risk?

Heme iron (as hemin or hemoglobin) has been shown to consistently increase colon cancer risk, as previously described. Sesink (2000) showed that a minimum of 0.16 μM of heme per gram of diet (as hemin) was required to increase fecal water concentration to 42 μM which induced fecal water cytotoxicity and increased the number of proliferating cells in the colon. In chapter 5, red meat was added to the high protein diet to provide twice the amount of heme iron (0.38μM heme/g diet) than the minimum amount required to induce toxic effects in colon. Although the animals fed a high red meat diet were supplied with high amounts of dietary heme iron, a majority of the heme iron was absorbed and only a small amount, 25.1 μM, reached the colon. This amount is considerably less than the minimum amount of heme iron shown to be cytotoxic and promotional of proliferation in the colon (Sesink, 2000).

A factor contributing to the high red meat diet showing no significant increase in colon cancer risk may be the difference in absorption properties of heme iron, which changes depending on the dietary form in which it is administered. Less heme iron reaches the colon when it is supplied in the form of red meat rather than in the purified form. Only 25% of heme iron from red meat has been found to reach the colon, whereas 97% of

hemin and 88% of hemoglobin passes into the colon (Schwartz and Ellefson, 1985; Sesink et al, 1999).

These findings suggest that a high red meat diet does not significantly increase the concentration of heme iron in the fecal water to a level which has been shown to stimulate tumor growth, or increase fecal water cytotoxicity and proliferation in the colon.

7.2 Whey protein and colon cancer risk

There is increasing evidence that whey protein may protect against colon cancer in comparison to other dietary protein sources. In carcinogen-induced colon tumor studies in rats, whey protein in comparison to red meat, soy bean meal and/or casein inhibited tumor expression (Bounous et al, 1988; Papenburg et al, 1990; McIntosh et al, 1995; Hakkak et al, 2001). The effect of whey protein and its concentration on ACF expression was examined in Chapter 5. The addition of whey protein to a diet (32% protein) reduced the expression of single ACF in the proximal colon by approximately 50% in comparison to the addition of red meat (16% and 32% protein). This finding supports a previous study that showed a similar protective effect against colon tumor expression for whey protein in comparison to red meat (McIntosh et al, 1995).

Various mechanisms have been postulated for a possible role of whey protein inhibiting carcinogenesis. These include the ability for whey protein to stimulate the immune system (Bounous et al, 1988; Bounous et al, 1989), to increase the concentration of

glutathione in plasma and liver (McIntosh et al, 1995; Lothian et al, 2000; Micke et al, 2001; Micke et al, 2002), to act as an antioxidant in-vitro (Colbert and Decker, 1991; Tong et al, 2000), to bind bile acids *in vitro* (Kruidenier et al, 1985) and reduce fecal fat (McIntosh et al, 1995). In Chapter 5, the high whey protein diet had a profound influence on body weight gain of the rats, reducing it from about 14 g/wk/rat (32% RM fed rats) to 8.7 g/wk/rat (32% WPC fed rats). This reduction in weight gain may be due to reduced plasma insulin, growth hormone and/or IGF-I and its binding proteins. These hormones stimulate growth and are widely believed to play a central role in carcinogenesis due to their important role in the regulation of cell cycle (Aaronson et al, 1990). Epidemiological studies have shown that individuals have an increased risk of colon cancer if they had NIDDM (Hu et al, 1999), a high basal secretion of IGF-I, a low plasma concentration of IGF-I binding protein (Ma et al, 1999) or if they have acromegaly, a condition characterized by an excess of growth hormone and IGF-I (Klein et al, 1982; Ituarte et al, 1984). Animal studies support an association between growth factors and their promotion of colon cancer risk (Bruce et al, 2000).

7.3 Metabolic effect of dietary proteins

7.3.1 Protein density

In Chapter 6, a high protein diet, irrespective of protein type was effective in reducing the storage of visceral, subcutaneous and carcass fat in the mature rat. The reduction in body fat for animals consuming a high protein diet was partially explained by a decrease in energy intake. This finding is in agreement with previous studies in the rat which have shown that on a weight for weight basis, protein is more satiating than carbohydrate and

fat (Jean et al, 2001; Bensaid et al, 2003). Thus animals on a high protein diet consume less energy which may otherwise be stored as fat. The reduction in fat storage for animals consuming the high protein diet may occur at the increased metabolic cost of digesting protein. Energy expenditure has been shown to increase by up to 160% following a high protein meal in comparison to a high carbohydrate or fat meal (Crovetti et al, 1998). Although the thermic effect of food is only a small proportion of daily energy expenditure, the change seen in the study by Crovetti et al (1998) in relation to the high protein diet over 3 meals could equate to a considerable increase (8.4% or 480kJ) in energy expenditure per day.

Another possible mechanism whereby the high protein diet may reduce body fat storage is by increasing the circulating concentration of thyroid hormones (T3 and T4). Thyroid hormones regulate metabolic rate and favour energy utilization through the mobilization and utilization of glucose and lipid stores. Layman et al (2003) showed that thyroid hormones were elevated following a high protein meal in comparison to a high carbohydrate diet. However, in Chapter 6 there was no difference detected in thyroid hormone concentration in fasted plasma samples from low or high protein fed animals. This indicates that thyroid hormone concentration may only be effected by protein density in the hours following consumption of the diet.

In Chapter 6, the high protein diet fed animals also had a reduced fasting plasma triglyceride concentration, which suggests that there is a reduction in VLDL production by the liver. This reduction in plasma triglyceride has been shown previously with high protein diets, where a reduction in body weight or visceral fat was also seen in human

subjects (Baba et al, 1999; Skov et al, 1999; Samaha et al, 2003). Another beneficial effect of the high protein diet was a sparing of lean body mass, whereby total muscle protein of the carcass increased and carcass fat decreased (Chapter 6). These changes in body composition which are predominantly caused by a reduction in energy intake, did not account for the reduction in IGF-I concentration in the plasma. Previous studies have shown that increasing the protein level in the diet did not effect circulating IGF-I concentration, which was only lowered in rats fed inadequate protein diets (Dardevet et al, 1991; Hirschberg and Kopple, 1991; Filho et al, 1999; Noguchi, 2000). This is inconsistent with the results in Chapter 6, showing a reduction in IGF-I concentration on the high protein diet. However, the mechanism for this reduction is not clear and requires further investigation.

In the scientific literature there is consistent evidence that fasting plasma insulin in glucose homeostasis regulation is affected by changes in visceral fat mass (Lemieux et al, 1996). The current study showed that increasing protein density in the diet reduced fat storage, but did not affect fasted plasma insulin, glucose or the insulin/glucose ratio. This may be explained by the differing actions of whey protein and red meat on metabolism.

7.3.2 Whey protein

Previously it has been shown that feeding moderate levels of whey protein to rats reduced their growth rate and final body weight in comparison to casein (Hakkak et al, 2000; Minehira et al, 2000; Badger et al, 2001). The effect of whey protein on growth rate and final body weight was investigated in Chapter 5. It was shown that an increased density of whey protein in the diet was inversely related to weight gain such that for every one

per cent increase in protein, there was a 0.15g decrease in weight gain per week. As a result, final body weight decreased with increasing whey protein in the diet. In Chapter 6, a follow up study was conducted to further examine the effect of whey protein on fat storage, carcass composition and fasted plasma metabolite and hormone concentration in insulin resistant rats. It was found that increasing the density of whey protein in the diet was associated with a reduction in body weight gain. The whey protein diet also improved insulin sensitivity in comparison to the red meat group that was reflected by a reduction in the fasting plasma insulin concentration. These changes may explain the reductions seen in visceral fat for the 32% whey protein fed rats compared to the low protein fed rats, as visceral obesity correlates strongly with insulin resistance (Lemieux et al, 1996; Yamashita et al, 1996; Bonora, 2000).

7.3.3 Red meat

Epidemiological studies show some evidence for a positive relationship between red meat dietary patterns and body mass index (Maskarinec et al, 2000; Newby et al, 2003). However, the findings of these prospective studies are limited due to the difficulties in removing the effect of other dietary components so as to specifically examine the effect of red meat on body mass index. Animal studies showed that red meat increased body weight in comparison to whey protein (McIntosh et al, 1995; Lai et al, 1997). Increasing the density of red meat in the diet was also associated with weight gain in rats (Chapter 5) (Parnaud et al, 1998). When the study described in Chapter 5 was repeated using older rats with insulin resistance (Chapter 6), rats consuming the high red meat diets grew at the same rate as low red meat fed animals. As the high red meat fed animals consumed

significantly less energy than low red meat fed animals it was not clear why they grew at the same rate.

7.4 Summary

The heme iron content of red meat has been proposed as a possible way that red meat may increase colon cancer risk. In this thesis it was shown that a high heme iron diet in the presence of polyunsaturated fat but not saturated fat (AMF) increased colon cancer risk by increasing the cytotoxicity and genotoxicity of fecal water. However, when a high red meat and polyunsaturated fat diet was fed to rats it did not increase ACF expression in comparison to a low red meat diet. The lack of an effect may be due the increased absorption of heme from red meat which leaves insufficient heme in the colon to increase colon cancer risk. This research suggests that heme iron in a high red meat diet is not likely to be a contributing factor to increase colon cancer risk.

In comparison to red meat, whey protein may be an alternative protein source that could be incorporated into the diet to protect against colon cancer risk. The high whey protein fed animals had significantly less ACF in the proximal colon in comparison to the moderate and high red meat fed animals. A possible mechanism whereby whey protein may inhibit colon carcinogenesis is by improving insulin sensitivity through reducing weight gain and body fat deposition. The added benefit of whey protein is that it may be an effective dietary factor to assist reducing the risk of NIDDM, insulin resistance or obesity. In addition, whey protein may also improve disease management for individuals who have either one or more of the conditions.

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APPENDIX 1. Journal publication arising from work completed in Chapter 5

Belobrajdic, D.P., McIntosh, G.H. & Owens, J.A. (2003) Whey proteins protect more than red meat against azoxymethane induced ACF in Wistar rats.
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APPENDIX 2. Journal publication arising from work completed in Chapter 6

Belobrajdic, D.P., McIntosh, G.H. & Owens, J.A. (2004) A high-whey-protein diet reduces body weight gain and alters insulin sensitivity relative to red meat in Wistar rats.
Journal of Nutrition, v. 134(6), pp. 1454-1458

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