An Assessment of Pyridoxine as a Biological Response Modifier During Colon Carcinogenesis

by

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Abstract

The main objective of this proposal was to investigate the effect of vitamin B_6 on colon carcinogenesis *in vivo*. Two *in vivo* studies were conducted to determine the role of vitamin B_6 as a biological modifier of colon carcinogenesis. It is hypothesized that vitamin B_6 may serve as an antioxidant *in vivo* and will modulate colon carcinogenesis. In the first study, a 2X3 factorial experimental design was used to determine if three different levels of vitamin $B_{6,}$ classified as low, normal and high in conjunction with two different levels of protein intake, classified as normal or high, will affect post-initiation stages of colon carcinogenesis, in Sprague-Dawley rats. Male Sprague-Dawley male were injected with azoxymethane for two weeks (15mg/kg/week) and then one week later they were allocated to different dietary treatment groups. After eight weeks, the effects of dietary treatment on hematological status, oxidative stress markers and antioxidant enzymes, as well as enumeration of preneoplastic lesions, aberrant crypt foci (ACF), were evaluated. The lowest level of vitamin B_6 intake with a high protein diet reduced the growth and development of ACF. Vitamin B_6 had no significant effect on the oxidative stress markers. The level of protein was an important variable in modulating the levels of 3-nitrotyrosine and 8-OH-DG which were lower in high protein groups than normal protein counterparts.

The objective of the second study was to investigate if a supraphysiological (5 fold higher than normal level) dosage of vitamin B_6 could have an antioxidant effect in a metabolically compromised state like obesity and thereby lower the risk of colon cancer. Female Zucker obese (Zk-OB) rats received normal (Zk-OBN, 7 mg/kg) or high (Zk-OBH, 35 mg/kg) vitamin B_6 (Pyridoxine-HCl) diets two weeks prior to, during and six weeks following injection with colon carcinogen AOM. The effects of supplemental vitamin B_6 on hematological status, oxidative stress markers and antioxidant enzymes, as well as enumeration of ACF were carried out. High intake of vitamin B_6 significantly lowered liver weights and plasma cholesterol compared to the normal intake ($p \leq 0.05$). Zk-OBH rats had significantly reduced number of ACF compared to Zk-OBN ($p \leq 0.05$). Hepatic GSH increased in the ZK-OBH group with a concomitant decrease in GPx activity. The findings

demonstrate that in Zucker Obese rats, a high B_6 intake augmented the antioxidant potential and decreased sensitivity to colon carcinogenesis. These findings suggest that high vitamin B_6 plays an important therapeutic role in the compromised state of obesity.

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For Mom and Dad

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Introduction

Colorectal cancer is a significant cause of death in the Western world. Epidemiological evidence indicates that nutrition plays a role in the modulation of colon carcinogenesis. In cancer prevention, it is important to understand which nutrients can have a protective effect and at which stages of pathogenesis. Epidemiological and experimental studies have alluded to the possibility that vitamin B_6 could reduce the risk of developing colon cancer.

Vitamin B_6, also known as pyridoxine, is a co-factor for nearly 100 enzymes. Pyridoxine is vital to cell function by being involved in amino acid metabolism including sulphur-containing amino acids. Vitamin B_6 has also been implicated in modulating lipid metabolism, downregulating steroid hormone receptors, serving to alleviate oxygen-free radicals and more recently, functioning as an antioxidant scavenger itself. Any of the described functions could easily be implicated in the carcinogenic process.

A limited amount of *in vivo* work has been conducted to determine the role of pyridoxine in carcinogenesis. What is not clear from the published work is if vitamin B_6 deficiency or low intake augments the risk of developing colon cancer. And, how does high intake of pyridoxine affect carcinogenesis? Moreover, oxidative cellular damage has been suggested to play a role in the development of colon cancer. It needs to be determined if a pharmacologic dose of pyridoxine is essential for its function as an antioxidant.

The ambiguities related to the biological role of vitamin B_6 were addressed in two studies. The primary objective of the research documented in this thesis was to “further assess the role of vitamin B_6 as a biological response modifier *in vivo*”.

The specific aims were to:

1. Investigate if low vitamin B_6 status augments colon carcinogenesis in Sprague-Dawley rats (Study 1).
2. Determine if a supraphysiological dose of vitamin B_6 will ameliorate the heightened risk of colon cancer in Zucker Obese rats (Study 2).

Hypothesis: Low vitamin B_6 intake will augment the risk of developing colon cancer in part by increasing oxidative stress *in vivo*.

To put this thesis in perspective, brief background information related to the biological activity of vitamin B_6 and pathogenesis of colon cancer is provided.

Chapter 1

Colon Cancer and the Potential of Vitamin B_6 as a Nutraceutical

1.1 Colon Carcinogenesis

Colorectal cancer is a significant cause of death in the Western world (Lipkin et. al., 1999). It may develop sporadically, induced by dietary or environmental agents, or as a result of inherited cancer-predisposition syndromes, familial polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) (Guanti and Bukvic, 2000). Colon cancer arises from a series of histopathologic and molecular changes that transform normal colonic epithelial cells into aberrant crypt foci, followed by adenomatous polyps and finally carcinoma (Janne and Mayer, 2000). In 1988, Vogelstein and others published a report that describes the accumulation of genetic events which include mutations to proto-oncogenes and tumor suppressor genes that occur during neoplastic progression (Figure 1) (Vogelstein et. al., 1988). These changes add a proliferative advantage to the colonic epithelial cells lining the lumen, contributing to the development of the malignant phenotype (Alrawi et. al., 2006 and Gryfe et. al., 1997).

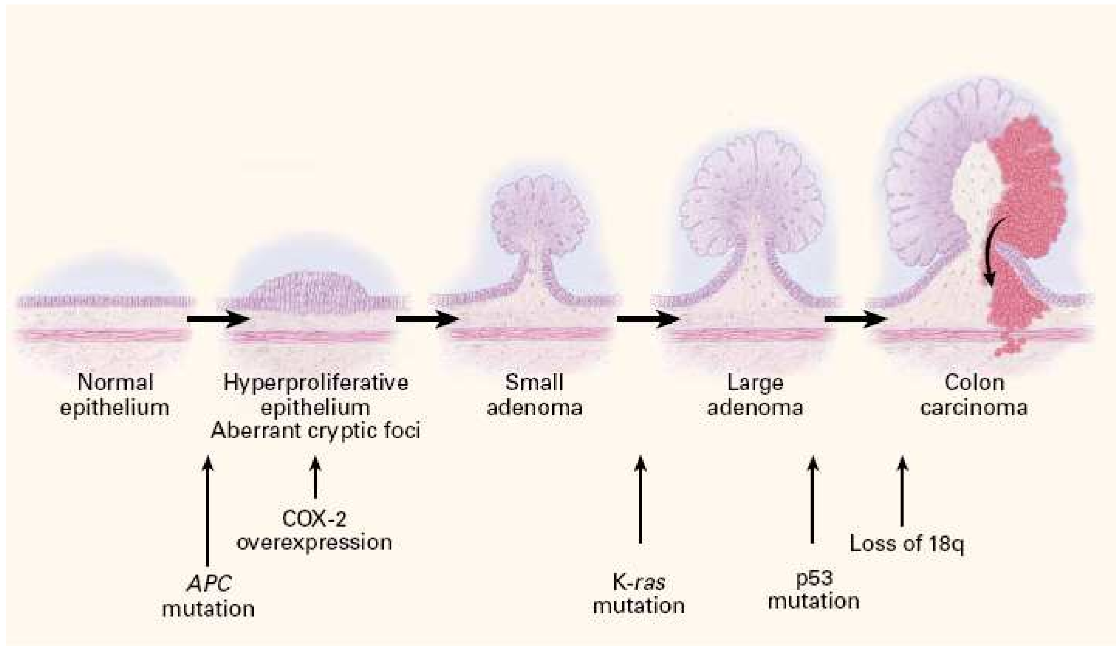

Figure 2. Vogelstein model of carcinogenesis

Progression of colon carcinogenesis from normal epithelium to carcinoma. Morphological changes correspond to sequential genetic alterations as proposed by Vogelstein et. al.,1988. Image adapted from Takhar et al. 2004.

1.1.1 Aberrant Crypt Foci (ACF)

Aberrant crypt foci were first described in 1987 by Bird in carcinogen-treated mice (Bird, 1987). ACF is an accepted and relevant model in the study of the progression of neoplastic transformation in colonic epithelial cells. Genetic alterations including K-ras, APC and p53 mutations, which are a trademark of colon cancer, have been identified in ACF, supporting their role as preneoplastic markers (Smith et. al., 1994 and Pretlow et. al., 1993). As biomarkers for colon carcinogenesis, enumeration of ACF permits rapid and inexpensive analysis of the affect of preventative agents in disease progression.

ACF are observed under the microscope once the colon mucosal surface has been stained with indigo carmine or methylene blue (McLellan, 1991). Compared to normal crypts, ACF appear raised from the surrounding mucosa with a thicker epithelial lining and oval or slit-like lumens (McLellan, 1991). They are visualized singly or grouped. Crypt multiplicity is an important parameter for evaluating ACF progression. ACF can be categorized as primal (1-3 crypts), intermediate (4-6 crypts) or advanced (7+ crypts) (Figure 2). Studies indicate that ACF density increases from the proximal colon toward the distal end which is line with the location of colorectal cancer (Roncucci et. al., 1993).

ACF can be induced experimentally in the rat colon with subcutaneous administration of azoxymethane, a colon-specific carcinogen. The appearance of ACF is visualized microscopically within as little as two weeks of azoxymethane administration (Bird, 1989).

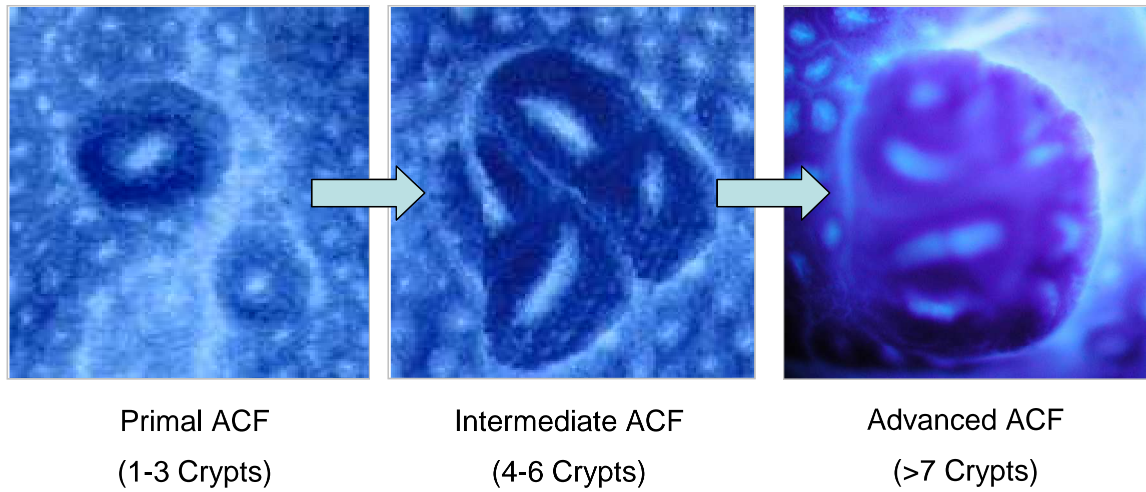

Figure 3. Growth features and classification of aberrant crypt foci (ACF)

1.2 Colon Cancer and Obesity

There is mounting epidemiological evidence that suggests obesity is associated with colon cancer (Gunter and Leitzmann, 2006). Obesity is characterized by a state of insulin resistance which is defined as the impaired ability of a cell to respond to insulin secreted for normalizing plasma glucose levels (Leonard et. al., 2005). Insulin resistance generally results in hyperglycemia, a condition that leads to diabetes, and has been implicated as a key factor in the development of colon cancer (Gunter and Leitzmann, 2006). Obesity is also characterized as a proinflammatory state believed to be induced by excessive production of storage lipids and high-circulating levels of glucose. Due to the individual relationships of obesity and chronic inflammation with colorectal cancer, and the role of obesity in causing inflammation, it is hypothesized that inflammation in obesity may be a key promoter of colorectal carcinogenesis (Gunter and Leitzmann, 2006).

In animals, the relationship between colon cancer and obesity can be investigated using Zucker Obese rats (Zk-Ob) and their lean counterparts (Zk-Ln). Zucker Obese rats inherit obesity as an autosomal Mendelian recessive trait for the leptin receptor (fa/fa, homozygous for nonfunctional receptors) (Bray, 1977). Leptin is a protein hormone that is secreted from adipocytes and circulates in the plasma (Pellymounter et. al., 1995). Leptin functions to regulate feeding and energy expenditure by sending signals to the hypothalamus to suppress appetite (Pellymounter et. al., 1995). As a result of the leptin receptor mutation, Zucker Obese rats have increased food consumption, hyperinsulinemia, enlarged fat cells and altered function of the endocrine system (Bray, 1977).

The physiological state of Zucker Obese rats provides an excellent model for investigating the role of vitamin B_6 in modulating oxidative stress and colon carcinogenesis and its potential as a therapeutic compound in carcinogenesis.

1.3 Vitamin B_6 and Colon Cancer

There is a significant amount of experimental evidence suggesting that dietary factors play an important role in the modulation of colorectal cancer (Lipkin, 1999). Epidemiological studies indicate an inverse relationship between vitamin B_6 intake, plasma PLP and the risk of colon cancer. A nested case-control study reported a statistically significant inverse association between plasma PLP concentrations and the risk of both colorectal and colon cancer (Wei et. al., 2005).

Several *in vitro* studies have shown that high levels of vitamin B_6 can suppress the growth of cancer cells (Disorbo and Litwack 1982 and Disorbo and Nathanson, 1983). More recently, animal models have been employed to investigate the chemopreventative capabilities of vitamin B_6 on the development of tumors. Komatsu et al. examined the effect of vitamin B_6 supplementation in the form of pyridoxine hydrochloride (PN HCl) in mice receiving azoxymethane injections over ten weeks (Komatsu et. al., 2001). Mice were terminated after 22 weeks. They compared the effect of 1, 7, 14 and 35 mg PN HCl/kg on the incidence and number of colonic tumors, cellular proliferation and apoptosis. Mice receiving 1 mg PN HCl/kg had a significantly higher number of adenomas. The number of adenocarcinomas in mice receiving 1 and 7 PN HCl/kg was four and three, respectively. Animals receiving 14 and 25 mg PN HCl/kg did not develop adenocarcinomas (Komatsu et. al., 2001). The minimum amount of pyridoxine required to suppress cellular proliferation was 7 mg PN HCl/kg. Cellular proliferation in the proximal colon was significantly lower for animals receiving 14 and 35 mg PN HCl/kg but did not differ among the three supplemented groups in the distal colon (Komatsu et. al., 2001). Supplemental vitamin B_6 was shown to have no effect on apoptosis in colonic cells. These results suggest that supplemental vitamin B_6 may have a preventative role in carcinogenesis.

1.4 Role of Oxidative Stress in Carcinogenesis

Oxidative cellular damage plays a significant role in the development of several human diseases including atherosclerosis, cancer and diabetes (Weisel, 2006). Respiration and glucose metabolism generate hydroxyl radicals and other reactive species that can cause a wide range of oxidative damage within the cell. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) include super oxide anion, hydrogen peroxide and nitric oxide. The reaction of ROS and RNS with DNA can have deleterious effects by introducing single- or double-stranded breaks, modifying purine and pyrimidine bases, or by creating DNA cross-links (Klaunig and Kamendulis, 2004). These types of DNA damage can lead to changes in transcriptional activation, induction of signal transduction pathways, replication errors and genomic instability (Klaunig and Kamendulis, 2004). All of these factors are implicated in carcinogenesis. Additionally, reactive oxygen species can also cause damage by lipid peroxidation, which involves the loss of electrons from the double bonds of polyunsaturated fatty acids. Since the cell membrane is heavily concentrated with polyunsaturated fatty acids, lipid peroxidation alters membrane integrity (Thomas et. al., 1995).

Non-enzymatic antioxidants and antioxidant enzymes function to counteract the effect of reactive oxygen species forming an antioxidant network (Figure 3). Since antioxidants interact with and regenerate other antioxidants within the network, a reduction of one of these species can have a significant effect on the balance and function of the system. The most effective antioxidant enzymes include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). Superoxide dismutase is a metalloprotein that dismutes superoxide anion (O^{2-}) forming hydrogen peroxide (H_2O_2) (Valko et. al., 2006). Catalase and glutathione peroxidase compete for H_2O_2 as a substrate in a decomposition reaction that yields water, thereby protecting the cell against oxidative damage. Non-enzymatic antioxidants include Vitamin C, Vitamin E, carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), and flavanoids (Valko et. al., 2006).

The non-enzymatic antioxidant, glutathione, is a tripeptide and a major soluble thiol-disulphide redox buffer of the cell (Thomas et. al., 1995). It is highly abundant in the cytosol, nuclei and mitochondria (Thomas et. al., 1995). The reduced form of glutathione is GSH and the oxidized form is referred to as glutathione disulphide (GSSG). GSH has many protective roles including scavenging hydroxyl radicals and singlet oxygen directly and serving as a co-factor for glutathione peroxidase (Valko et. al., 2006). The antioxidant capacity of GSH is due to the sulphur compound that can accommodate the loss of an electron. It can also regenerate many important antioxidants to their active forms: Vitamin C and E (Valko et al., 2006). Due to GSH's role in reducing oxidized products and regenerating other antioxidants, the ratio of GSH/GSSG is a good measure of oxidative stress in an organism.

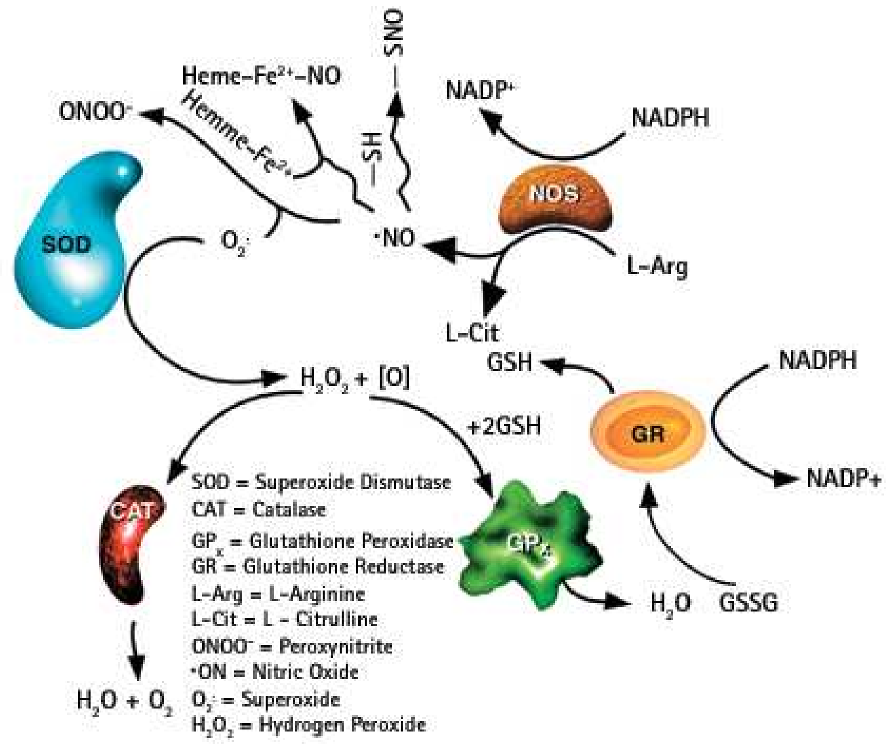

Figure 4. Antioxidant network (Calbiochem, 2006)

1.5 Physiological Role of Vitamin B_6

Vitamin B_6 is a water-soluble vitamin required for normal growth and development. It exists in three free forms which include pyridoxine (PN), pyridoxal (PL), and pyridoxamine. Each form can be converted to the active coenzymes, pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP), by phosphorylation at the 5' position in the liver (Leklem, 1999). The PLP form is covalently bound to enzymes *via* a Schiff base with the ε-amino group of lysine. Vitamin B_6 has many important functions that are complex and interrelated. Due to the reactivity of PLP with amino acids and nitrogen containing compounds, amino acid metabolism is the most recognized function (Leklem, 1988). PLP is also involved in gluconeogenesis, niacin formation, lipid metabolism, steroid hormone modulation, homocysteine conversion, and red blood cell metabolism and function. Additionally, both the nervous and immune systems require vitamin B_6 to function efficiently.

1.5.1 Relationship between Protein Intake and B_6 Requirement

The recommended daily intake for vitamin B_6 in adult males and females under the age of 50 is 1.3 mg/d (Food and Nutrition Board, 2005). Due to its broad role as a co-factor in many metabolic processes, adequate consumption is necessary. The requirement for vitamin B_6 is related to the level of dietary protein; high protein intake increases B_6 requirement (Okada et. al., 1998). PLP functions as a co-enzyme for transaminases that participate in the catabolism of all amino acids (Okada, 1971). These include transaminases L-alanine and L-aspartate. During high protein intake, the activities of liver transaminases are generally induced to compensate for increased catabolism of amino acids (Okada, 1971). However, in a vitamin B_6 deficient state, rats receiving high protein (70% casein) have significantly decreased activities of these hepatic transaminases (Okada, 1971). The animals have also been shown to develop fatty livers with increased levels of glycerides and cholesterol. Growth inhibition is also observed (Okada, 1971).

Studies indicate that when vitamin B_6 intake is adequate, high dietary protein favors retention of the vitamin in the liver. Conversely, during deficient intake of vitamin B_6, liver levels of PLP are inversely proportional to protein intake (Itoh and Okada, 1973 and Okada et. al., 1998). A study of men receiving adequate amounts of vitamin B_6 (1.6 mg) has shown that plasma concentrations of PLP and urinary 4-pyridoxic acid are also inversely related to protein intake (Miller, 1985). Okada et. al. also showed that the plasma B_6 levels of rats fed a 20% casein diet were higher than that of groups receiving 70% casein when there was an equal amount of B_6 in the diets (Okada, 1998).

1.5.2 Glutathione Production: Role of Vitamin B_6 in Homocysteine Metabolism

Homocysteine is a non-protein forming sulfur amino acid. Hyperhomocysteinemia is a condition that refers to elevated levels of homocysteine in the plasma and most notably leads to vascular disease. Levels of plasma homocysteine and its metabolism are influenced by genetic and dietary factors including methionine content and circulating folic acid, vitamin B_{12} and vitamin B_6 (Selhub, 1999). Homocysteine can be metabolized by one of two pathways: remethylation or transulfuration (Figure 4). Remethylation involves converting homocysteine to methionine, a reaction that is B_{12}-dependent in all tissues (Selhub, 1999). Transulfuration involves metabolizing homocysteine to cysteine by two PLP-dependent enzymes: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGS). Cysteine is an essential amino acid and the limiting reagent for glutathione synthesis (Davis et. al., 2005). CBS irreversibly catalyzes the condensation of homocysteine with serine to form cystathionine. Cystathionine is then converted to cysteine by CGS (Davis et. al., 2005).

Previous studies have reported that vitamin B_6 deficiency causes elevated levels of homocysteine in tissue and plasma. Other observed effects of vitamin B_6 deficiency include increased lipid peroxidation in plasma, kidney, heart, and liver tissues, and decreased activity of PLP-dependent CBS (Ravichandran and Selvam, 1990 and Cabrini et. al., 1998 and Cabrini et. al., 2005). In another study, vitamin B_6 deficiency resulted in decreased activity of CGS. Male Wistar rats consuming diets high in protein (70%

casein) and deficient in vitamin B_6 excreted unusually high amounts of cystathionine which appeared to be reflective of decreased cystathionine γ-lyase activity (Okada, 1974). Rats receiving high-protein diets with pyridoxine supplementation only had traces of the amino acid. Cumulatively, the results of previous studies suggest that vitamin B_6 deficiency should reduce glutathione synthesis as evidenced through elevated levels of homocysteine and decreased activities of CBS and CGS. As a result, reduced glutathione levels should translate into a decreased ability to normalize superoxide anion levels.

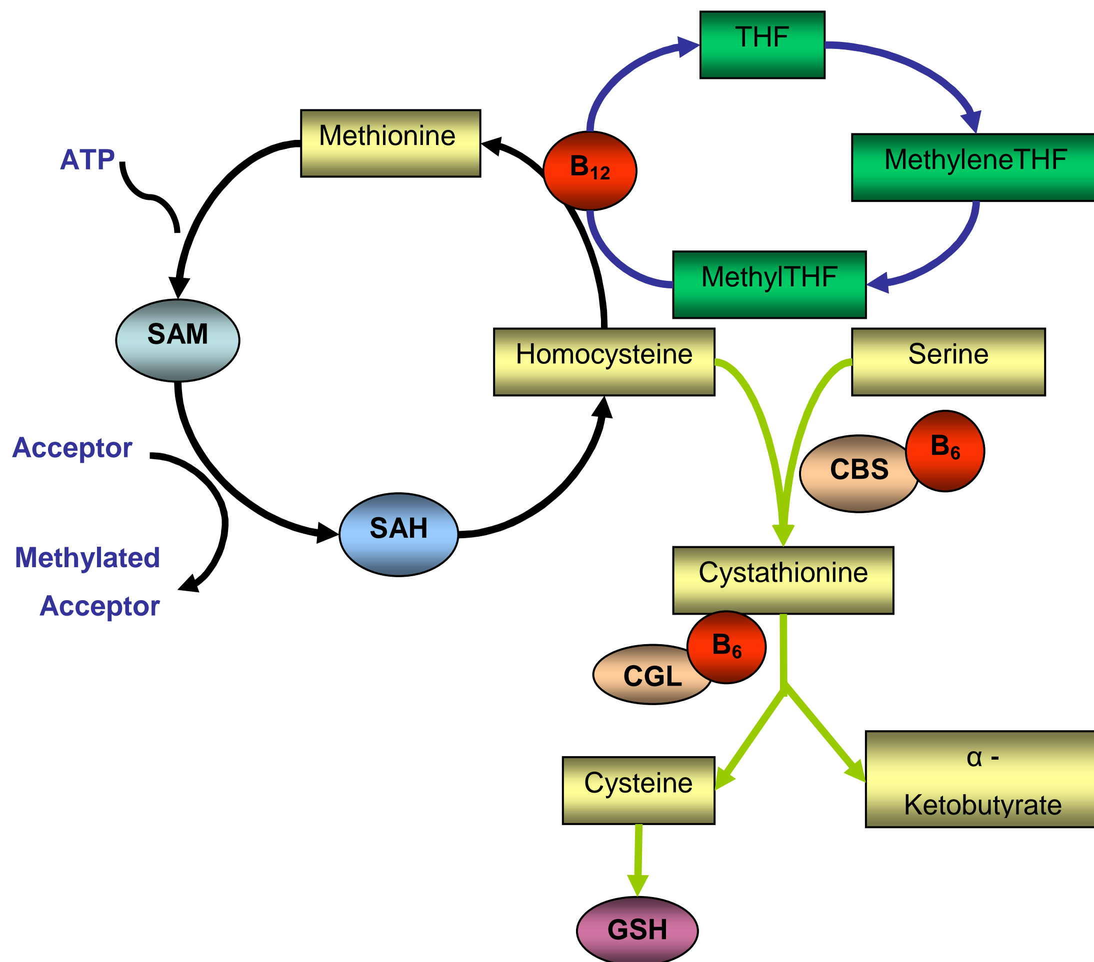

Figure 5. Homocysteine metabolism

The black arrow indicates remethylation while the green arrow indicates the transulfuration pathway. Methionine is converted to S-adenosyl-methionine (SAM), the main methyl donor in the body. SAM can be further converted to S-adenosyl-homocysteine (SAH) and then to homocysteine. Homocysteine metabolism to cysteine is catalyzed by two PLP-dependent enzymes: CBS and CGS. Additional Abbreviations: THF, tetrahydrofolate; MethylTHF, N-5-Methyltetrahydrofolate; MethyleneTHF, N-5-Methylenetetrahydrofolate. Adapted from Selhub 1999.

1.5.3 Role of Vitamin B_6 as a Modulator of Oxidative Stress

Dietary factors play a role in both promoting and preventing oxidative stress (Thomas, 1994). As described earlier, vitamin B_6 functions as a co-factor in glutathione synthesis thereby serving as a modulator of oxidative stress. More recently, vitamin B_6 has been proposed to have antioxidant properties by functioning as a scavenger for free radicals (Grimble 1997 and Kannan and Jain, 2004).

In 2004, Kannan and Jain proposed through a study using U937 monocytes cultured with PN and PLP, that vitamin B_6 might function as an antioxidant by scavenging oxygen radicals. Treatment of the U937 cell line with vitamin B_6 showed that higher concentrations significantly decreased the generation of ROS, lipid peroxidation, and H_2O_2-induced changes in mitochondrial membrane permeability (Kannan and Jain, 2004). Evidence suggests that phenolic compounds react faster with peroxy radicals than peroxy radicals can react with the lipids (Noguchi et. al., 1998). Studies indicate that functional groups such as hydroxyl and amine can also scavenge oxygen radicals (Noguchi et. al., 1998). Given the chemical structure of vitamin B_6 as a phenolic compound with both hydroxyl and amine groups substituted on the pyridine ring, Kannan and Jain hypothesized that, alone, it can function as an antioxidant. However, the exact chemical mechanism by which pyridoxine would scavenge oxygen radicals and inhibit lipid peroxidation has not yet been confirmed.

A physiological difference may exist between adequate consumption of vitamin $B_{6,}$ which ensures a balance in the antioxidant network, and supplementary intake to quench excessive production of reactive oxygen species. The cellular concentration of glutathione has a major affect on the balance of the antioxidant network. Consequently, it should be expected that compromised vitamin B_6 status, the result of reduced intake, will lead to decreased glutathione allowing reactive oxygen species to accumulate, translating to cancer promoting effects. In an obese state characterized by hyperglycemia, adequate consumption of vitamin B_6 may not be enough to counteract the physiological consequences of high glucose. In diabetic patients and *in vitro* studies, high levels of

glucose have been shown to generate superoxide radicals from the autoxidation of glucose, resulting in increased cellular lipid peroxidation and glycosylated hemoglobin (Jain and Lim, 2001). In the proposed role of an oxygen radical scavenger, supplementary vitamin B_6 may be necessary to normalize levels of ROS and enhance glutathione production and, in doing so, exert a protective effect against colon carcinogenesis.

Chapter 2

Materials and Methods

2.1 Materials

Unless otherwise stated all chemicals and reagents were purchased from Sigma Chemical Co., Mississauga, Ontario.

2.2 Animal Care

Sprague Dawley, Zucker Obese and Lean rats were procured from Charles River Canada (St-Constant, Quebec). Animals were housed in polypropylene cages lined with woodchip bedding and stainless steel wire mesh lids in the Animal Facility. Environmental conditions were controlled for temperature and humidity and maintained at 24°C and 55%, respectively, with a 12 hour light/dark photoperiod. Animals were housed in groups of three or four and given access to diet and water *ad libitum*. Animal care and all investigative procedures adhered to guidelines of the Office of Research Ethics, University of Waterloo (AUPP:04-17) and the Canadian Council of Animal Care.

2.3 Diets

All experimental diets (Harlan Teklad, Madison, WI) were based on the semi-synthetic AIN-93G diet containing 5% corn oil (wt/wt). In Study 1, diets were modified with respect to casein and pyridoxine hydrochloride (PN-HCl) (Appendix B, Table 1). In Study 2, diets were modified with respect to PN-HCl only (Appendix B, Table 2).

2.4 Complete Blood Count

At termination, a sample of blood from each animal was collected in a EDTA vacutainer tube (BD Vacutainer Systems, NJ) from a cardiac puncture using multiple sample Luer adapter and sent to Animal Health Laboratories (University of Guelph, Guelph, ON) for complete blood count. The complete blood count (CBC) was performed using the Adiva 120 Hematology System.

2.5 Biochemical Assessment

A sample of blood from each animal was collected in a heparinized vacutainer tube (BD Vacutainer Systems, NJ) from a cardiac puncture using a multiple sample Luer adapter and centrifuged for 10 min at 2000 rpm to separate plasma. Plasma samples were aliquoted and sent to Animal Health Laboratories (University of Guelph, Guelph, ON) for biochemical analysis using rat specific parameters.

2.5.1 Creatinine

Serum creatinine concentration is an accepted index for glomerular filtration rate and is used to assess renal function (Perrone et. al., 1992). Creatinine was measured using an enzymatic ultraviolet absorbance method. Creatinine is hydrolyzed by creatinine deiminase to ammonia and N-methylhydantoin (Fossati et. al., 1994). The ammonia produced combines with 2-oxoglutarate and NADPH in the presence of glutamate dehydrogenase (GLDH) to yield glutamate and $NADP^+$ (Fossati et. al., 1994). The reduction in absorbance at 340 nm caused by the oxidation of NADPH between two fixed time points is proportional to the amount of creatinine in the sample. Prior to the addition of creatinine deiminase, endogenous ammonia present in the sample is removed by the addition of GLDH.

2.5.2 Urea

Plasma urea was determined using a commercially available kinetic UV assay kit from Roche. Urea analysis is based on the enzymatic determination method described by Talke and Schubert (1965) that uses the coupled urease/glutamate dehydrogenase (GLDH) enzyme system. Urea is the final degradation product of protein and amino acid metabolism. In this experiment, urea is hydrolyzed by urease to form CO_2 and ammonia. The ammonia formed then reacts with α-ketoglutarate and NADH in the presence of glutatmate dyhydrogenase (GLDH) to yield glutamate and NAD^+. The decrease in absorbance due to the oxidation of NADH is measured kinetically.

2.5.3 High Density Lipoprotein-Cholesterol (HDL-C)

Plasma HDL-C was determined using a direct enzymatic colorimetric test from Roche. The automated method for direct determination uses polyethylene glycol (PEG)-modified enzymes and dextran sulfate. In the presence of magnesium sulfate, dextran sulfate selectively forms water-soluble complexes with low density lipoproteins (LDL), very low density lipoproteins (VLDL) and chylomicrons which are resistant to PEG-modified enzymes. Cholesterol esterase and cholesterol oxidase enzymes are coupled with PEG which results in selective catalytic activities toward lipoprotein fractions, with increasing reactivity in the order of HDL>VLDL and chylomicrons>LDL. Following the addition of PEG-modified enzymes and 4-amino-antipyrine in buffer, HDL-cholesterol esters are hydrolyzed by PEG-cholesterol esterase to form HDL-cholesterol and fatty acids. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to its corresponding ketone and hydrogen peroxide (H_2O_2). In the presence of peroxidase, H_2O_2 reacts with 4-amino-antipyrine and HSDA to form a purple dye. The intensity of the dye was directly proportional to the cholesterol concentration and was measured photometrically.

2.5.4 Cholesterol

Plasma cholesterol was determined using a commercially available enzymatic colorimetric test supplied by Roche (CHOD-PAP). In this enzymatic assay, cholesterol esters are hydrolyzed by cholesterol esterase (CE) to cholesterol, which is then oxidized by cholesterol oxidase (CO) to yield cholest-4-en-3-one and H_2O_2 co-products. The concentration of cholesterol is detected through the amount of H_2O_2 formed. Peroxidase catalyzes the oxidative coupling of phenol with 4-aminophenazone which reacts with H_2O_2 to give a red coloured quinone-imine. This is based on the Trinder (1969) reaction. The intensity of the dye was measured photometrically using the automated clinical analyzer.

2.5.5 Glucose

Plasma glucose was determined using an enzymatic colorimetric assay from Roche. In this experiment, glucose is oxidized by glucose oxidase (GOD) to gluconolactone in the

presence of atmospheric oxygen. Measurement of the amount of H_2O_2 produced is through the Trinder (1969) reaction. The hydrolysis of H_2O_2 by peroxidase is detected using phenol and the chromogen 4-aminophenazone which acts as an oxygen receptor, producing a red colored compound known as 4-(p-benzoquinone-monoinino)-phenazone. The optical density was measured at 505 nm using a spectrophotometer to give the concentration of glucose.

2.5.6 Triglycerides

The level of plasma triglycerides was determined using a commercially available colorimetric enzyme kit from Roche (GPO-PAP). In this experiment, lipoprotein lipase from microorganisms is used to hydrolyze triglycerides to glycerol. Glycerol kinase (GK) then phosphorylates glycerol forming glycerol-3-phosphate. Glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate and H_2O_2. The H_2O_2 reacts with 4-aminophenazone and 4-cholorophenol under the catalytic action of peroxidase to form a red dye, 4-(*p*-benzoquinon-monoimino)-phenazone. The intensity of dye was measured using Roche clinical chemistry analyzers.

2.5.7 Total Bilirubin

Measurement of bilirubin is used to detect liver disease, hemolytic anemia and to evaluate the degree of jaundice. Total bilirubin in plasma was quantitatively determined using the Roche automated clinical chemistry analyzer. Plasma samples are mixed with a reagent that contains sodium acetate buffer, sulfamic acid, a surfactant and solubilizer. The surfactant is added to accelerate the reaction and avoid protein precipitation. Under strongly acidic conditions (pH 1-2), total bilirubin is rapidly coupled with a diazonium ion that is later added to the mixture, forming azobilirubin. The intensity of the color from azobilirubin was measured photometrically and was proportional to the concentration of total bilirubin.

2.5.8 Aspartate Aminotransferase (AST) Analysis

AST activity in plasma is determined using the Roche automated clinical chemistry analyzer. In this experiment, AST catalyzed the reaction between α-ketoglutarate and L-aspartate, forming L-glutamate and oxaloacetate. The increase in oxaloacetate was determined in an indicator reaction catalyzed by malate dehydrogenase (MDH) where NADH was oxidized to NAD^+. The rate of NADH oxidation was photometrically determined and was directly proportional to the rate of formation of oxaloacetate, thereby indirectly giving AST activity.

2.5.9 Alanine Aminotransferase (ALT) Analysis

Determination of ALT activity was performed according to a standardized method recommended by the International Federation of Clinical Chemistry using a Roche automated clinical chemistry analyzer. To prevent competing reactions with NADH, plasma samples were pre-incubated with a TRIS buffer that contains L-alanine, NADH and lactate dehydrogenase (LDH). In this experiment, ALT catalyzed the reaction between α-ketoglutarate and L-alanine. The pyruvate that was produced from this reaction was subsequently measured in an indicator reaction which was catalyzed by LDH. In the indicator reaction, NADH was oxidized to NAD^+. The rate of decrease in NADH was photometrically measured and was directly proportional to the rate of production of pyruvate and thereby giving the rate of ALT activity.

2.5.10 Creatine Kinase (CK)

CK activity was measured using the method proposed by Szasz (1979) and was quantitatively determined using a Roche automated clinical analyzer. CK was rapidly inactivated by oxidation of sulfhydryl groups in the active center. N-acetylcysteine (NAC) was added to reactivate the enzyme. Diadenosine pentaphosphate (ATP) and AMP were added to prevent interference by adenylate kinase. CK catalyzes the phosphorylation of ADP in the presence of creatine phosphate, to form adenosine triphosphate (ATP) and creatine. Hexokinase (HK) in the presence of magnesium ions catalyzed the phosphorylation of glucose by the ATP formed to produce glucose-6-phosphate. Glucose-6-phosphate dehydrogenase oxidized glucose-6-phosphate with

simultaneous reduction of the coenzyme nicotinamide adenine dinucleotide phosphate (NADP) to give NADPH and 6-phosphogluconate. The rate of NADPH formation was measured at 340 nm and was directly proportional to the activity of CK in the sample.

2.5.11 Total Protein

Total protein in plasma was analyzed using a colorimetric assay kit available from Roche. Plasma samples were mixed with copper in an alkaline solution. Divalent copper reacted with protein peptide bonds to form a purple coloured biuret complex. The color intensity was directly proportional to the protein concentration which was determined photometrically using a Roche automated clinical chemistry analyzer.

2.5.12 Albumin

The concentration of plasma albumin was colorimetrically determined using an endpoint method and quantified using a Roche automated clinical chemistry analyzer. Plasma samples were treated with a citrate buffer containing bromocresol green (BCG). At a pH=4.1, albumin displays a cationic character that enables it to bind with bromocresol green (BCG) to form a blue-green complex. The change in absorbance at 628 nm correlates with the concentration of albumin.

2.6 Pyridoxal 5'-Phosphate Measurement

The concentration of plasma PLP was determined within the laboratory of Dr. Jim House at the University of Manitoba. Plasma PLP concentrations were quantitatively determined using a commercially available radioenzymatic assay kit (ALPCO, Windham, NH, Vitamin B_6 PLP ^{3}H-REA). Due to the sensitivity of PLP to light, measurement was performed under protection from direct light exposure. The kit follows the principle that ^{3}H-tyrosine is decarboxylated by tyrosine apodecarboxylase (Y-apoDC), a vitamin B_6 dependent enzyme from *Streptococcus faecalis*. The activity of tyrosine apodecarboxylase is dependent on the amount of PLP present in the sample. The resulting compound that was produced, ^{3}H-tyramine, was selectively extracted in a scintillation

cocktail and was measured by liquid scintillation counting. The inter- and intra-assay coefficients of variation were < 10% and <5%, respectively.

2.7 Homocysteine and Cysteine Measurement

Measurement of plasma total homocysteine and cysteine was determined within the laboratory of Dr. Jim House at the University of Manitoba. Thiol measurement was determined using the reverse phase high performance liquid chromatography (HPLC) method of Araki and Sako (1987), with modifications as suggested by Gilfix et. al. (1997). Plasma samples were incubated with tris-carboxyethylphosphine (TCEP; Fisher Scientific, Nepean, ON) to reduce protein-bound and oxidized forms of homocysteine, followed by derivatization with 7-fluorobenzofurazan-4-sulfonic acid ammonium salt (SBD-F; Sigma Chemical Co., Oakville, ON). The fluorescent thiol derivatives were separated on a Waters C-18 column (5 μM, 4.5 x 250 mm; Waters Canada, Mississauga, ON) using isocratic elution (98% 0.1 M acetate, pH 5.5: 2% methanol) by means of a Shimadzu HPLC system (Man-Tech Associates, Guelph, ON) complete with autoinjector and fluorescence detector (excitation λ = 385 nm; emission λ = 515 nm). Concentrations of total homocysteine and cysteine were determined through the use of an external standard curve, and the inter- and intra-assay coefficients of variation were < 2%.

2.8 Plasma 3-Nitrotyrosine and 8-OH-DG Quantification

Plasma was assayed for levels free 3-nitrotyrosine and 8-hydroxy-2-deoxyguanosine (8-OH-DG) at the Ottawa Health Research Institute (Ottawa, ON). Analyses were carried out via a novel, highly sensitive non-radioactive HPLC method allowing simultaneous measurement of multiple oxidative stress markers (Kumarathasan and Vincent, 2003). The analytical precision values are 95% for HPLC Coularray.

2.9 Colon Preparation

The colon from each animal was removed and flushed with ice-cold saline solution and placed on a cold plate set at 4°C. A longitudinal cut was made from the rectal to the

ceacal end. Colons intended for morphological analyses were spread flat between two filter papers and fixed in 10% neutral buffered formalin.

In the Sprague-Dawley study, four colons in each group had a small section of mucosa scraped from each end (4 cm of proximal and 2 cm of distal end). The mucosa was frozen in liquid nitrogen and stored at -80°C for biochemical analyses.

2.9.1 Aberrant Crypt Foci (ACF) Enumeration

Enumeration of ACF was conducted according to the method described by Bird (1987). Fixed colons were cut into 6 cm sections and stained with 0.2% methylene blue stain for 4 minutes. Stained sections were placed mucosal side up on a microscope slide and observed through a light microscope. ACF were enumerated in 2 cm sections moving from the proximal to caecal end. The variables used to assess the aberrant crypts were occurrence and multiplicity. ACF were classified as primal (1-3 crypts), intermediate (4-6 crypts) or advanced (7+ crypts).

2.10 Preparation of Whole Extract from Liver Tissue

One gram of liver tissue stored at -80°C was sectioned and added to 3 mL of ice-cold RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.25% Sodium Deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF) with freshly added protease inhibitors (1µg/mL of Aprotinin, Leupeptin, Trypsin Inhibitor, Sodium Orthovandate) and then homogenized in ice using the PT2100 Polytron homogenizer. The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C. The top lipid layer was removed and the supernatant was collected and aliquoted into pre-chilled eppendorf tubes and stored in -80°C for further analysis.

Samples of whole extract from liver tissue were used for analysis of cholesterol, glutathione, and nitrite.

2.11 Liver Cholesterol Assay

The Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR) provides a simple fluorometric method for the sensitive measurement of cholesterol using a fluorescence microplate reader. In this enzymatic assay, cholesterol esters were hydrolyzed by cholesterol esterase (CE) into cholesterol, which was then oxidized by cholesterol oxidase (CO) to yield the corresponding ketone and H_2O_2. This enzymatic method for assaying cholesterol is based on the measurement of H_2O_2 by way of horseradish peroxidase (HRP)-coupled oxidation of Amplex Red, an H_2O_2-sensitive probe. Amplex Red reagent reacted with H_2O_2 to produce highly fluorescent resorufin.

To measure cholesterol, 50 µg of liver homogenate was analyzed with the Amplex Red cholesterol fluorescence assay kit according to the manufacturer's instruction. In brief, the assay was conducted in a 96-well microplate using a total of 100 µl reaction volume per well. Reagents added to reaction mixtures included 300 µM Amplex Red, 2 U/mL HRP, 2 U/mL cholesterol oxidase and 0.2 U/mL cholesterol esterase. The reaction mixtures were incubated at 37°C for 30 mins, and the fluorescence intensities were measured (excitation $\lambda = 560 \pm 10$ nm; emission $\lambda = 590 \pm 10$ nm) to determine cholesterol concentration.

2.12 Colorimetric Determination of Glutathione

The QuantiChrom Glutathione Assay Kit (BioAssay Systems, Hayward, CA, DIGT-250) was used to measure reduced glutathione in plasma and hepatic tissue samples of Sprague-Dawley rats. The simple and direct method used 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to detect reduced glutathione. A reaction between DTNB and reduced glutathione formed a yellow product. The optical density of the yellow product measured spectrophotometrically at 412 nm, was proportional to sample glutathione concentration.

The assay was conducted in a clear bottom 96-well plate using a final reaction volume of 300 µl per well. The concentration of reduced glutathione in liver tissue was assayed using hepatic tissue samples homogenized in RIPA buffer. Equal volumes of sample (plasma or liver extract) and sodium tungstate/DTNB reagent were mixed in eppendorf tubes and centrifuged at 14,000 rpm for 5 min. The supernatant for each sample was

transferred into the wells in duplicates and 100 µl of phosphate buffer was added to stabilize the colour produced in the reaction. The reaction mixtures were incubated at room temperature for 25 min and the optical density was measured at 412 nm. The inter-assay coefficient of variation was <2%.

2.13 Colorimetric Determination of Nitrite (NO_2^-)

Nitrite (NO_2^-) is a stable oxidation product of nitric oxide (NO). The QuantiChrom™ Nitric Oxide (NO) Assay kit (BioAssay Systems, Hayward, CA, DINO-250) follows the two step Griess method (1879) for colorimetrically determining the concentration of nitrite.

The assay was conducted in a clear bottom 96-well plate using a final reaction volume of 200 µl per well. Sample preparation included a two-step deproteination. In the first step, 100 µl of hepatic tissue homogenized in RIPA buffer or plasma was mixed with 80 µl of 75 mM $ZnSO_4$. The mixture was centrifuged at 14,000 rpm for 5 min. The supernatant was then transferred to eppendorf tubes containing 120 µl 55 mM NaOH for a second deproteination. The mixture was centrifuged at 14,000 rpm for 5 min and the supernatant was mixed with glycine buffer. Reduction of NO_3^- was achieved by the addition of 3 copperized cadmium granules to each sample/glycine mixture and followed by a 15 min incubation period at room temperature. Duplicate volumes of each sample were transferred into wells. Sulphanilic acid was added to react with NO_2^-, forming a diazonium salt. In a second step, 50 µl of *N*-(1-naphthyl)ethylenediamine was added to react with the diazonium ion forming a chromophoric azo. The reaction mixtures were incubated for 5 min at room temperature and the optical densities were measured at 540 nm. The inter-assay coefficient of variation was <8%.

2.14 Superoxide Dismutase (SOD) Assay

The activity of SOD in liver tissue was assayed using a commercially available kit (Cayman Chemical Ann Arbor, MI, Catalog No. 706002). This kit employs tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine.

2.14.1 Sample Preparation

A 1 g section of liver tissue from each Zucker Obese and lean rat and a 0.5 g section from each Sprague-Dawley animal was mixed with 7 ml of cold buffer (20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose) and homogenized in ice using the PT2100 Polytron homogenizer. Homogenates were centrifuged at 1,500 g for 5 min at 4°C. The supernatant was separated from cell debris and aliquoted in chilled eppendorf tubes. The protein concentration of each sample was determined using the Bradford method (1976) and samples were stored in -80°C for the assay.

2.14.2 Assay

The SOD assay was conducted in a 96-well microplate using a total of 230 µl reaction volume per well and each sample was assayed in duplicate. Each well was loaded with 200 µl of radical detector and 10 µl of undiluted Sprague-Dawley liver sample. In the Zucker Obese study, liver samples were diluted 10x with sample buffer and then mixed with radical detector. Reactions were initiated through the addition of 20 µl of xanthine oxidase to each well. The reaction mixtures were incubated on a plate shaker at room temperature for 20 min and the absorbance was measured at 450 nm using a plate reader. The intra- and inter-assay coefficients of variation were 3.2% and 3.7%, respectively.

2.15 Glutathione Peroxidase (GPx) Assay

GPx activity was assayed using a commercially available kit (Cayman Chemical Ann Arbor, MI, Catalog No. 703102). The procedure was based on the Paglia and Valentine method (1967) and indirectly measured GPx activity in a reaction coupled with glutathione reductase (GR). Upon reduction of hydroperoxides by GPx, oxidized glutathione (GSSG) was recycled to its reduced state by GR and NADPH. The production of $NADP^+$ was accompanied by a decrease in absorbance at 340 nm.

2.15.1 Sample Preparation

A 1 g section of liver tissue from was mixed with 10 ml of cold buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT) and homogenized in ice using the PT2100 Polytron homogenizer. Homogenates were centrifuged at 10,000 g for 20 mins at 4°C. The supernatant was separated from cell debris and aliquoted in chilled eppendorf tubes. The protein concentration of each sample was determined using the Bradford method (1976) and samples were stored in -80°C for the assay.

2.15.2 Assay

The assay was conducted in a 96-well microplate using a total of 190 µl reaction volume per well. Liver homogenate samples were diluted 10x using sample buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA and 1 mg/ml BSA). Equal volumes of 20 µl of liver homogenate were added to each well. To diluted sample, 100 µl of assay buffer and 50 µl of co-substrate were added. Reactions were initiated with the addition of cumene hydroperoxide substrate. The reaction mixtures were shaken for a few minutes and the decrease in absorbance was continuously monitored spectrophotometrically at 340 nm for a period of 6 min. One unit of GPx activity was expressed as the amount of enzyme that would catalyze the oxidation of 1.0 nmol NADPH to $NADP^+$ per minute at 25°C. The intra- and inter-assay coefficients of variation were 5.7% and 7.2%, respectively.

2.16 Catalase (CAT) Activity

Hepatic CAT activity was assayed using the commercially available Catalase Assay Kit (Catalog No. 707002, Cayman Chemical, Ann Arbor, MI). The procedure was performed according to the Johansson and Borg method (1988) which exploits the enzyme's peroxidatic function in the presence of methanol. In this experiment, the substrate methanol functions as an electron donor for CAT, generating 2 molecules of water and formaldehyde. The amount of formaldehyde generated was measured spectrophotometrically at 540 nm by reaction with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald), a chromogen.

2.16.1 Sample Preparation

A 0.5 g section of liver from was homogenized in 5 ml of buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) using the PT2100 Polytron homogenizer. The homogenized sample was centrifuged at 10,000 g for 15 minutes at 4°C and the supernatant was isolated for protein concentration and CAT activity determination.

2.16.2 Assay

Liver homogenate samples were diluted 400x in diluted sample buffer (25 mM potassium phosphate, pH 7.5, containing 1 mM EDTA and 0.1% BSA). To each well, 100 µl of diluted assay buffer, 30 µl methanol and 20 µl of sample was added. Reactions were initiated by the addition of 20 µl of dilute H_2O_2 to each mixture and incubated for 20 mins at room temperature. Subsequently, 30 µl of potassium hydroxide was added to terminate the reaction and 30 µl of Purpald was added to each well. The reaction mixtures were incubated for 10 mins at room temperature on a shaker. Finally, 10 µl of potassium periodate was added to each well and the plate was incubated for 5 min at room temperature. The optical density of each well was read at 540 nm using a plate reader. The inter-assay coefficient of variation was 9.9%.

2.17 Statistical Analysis

Statistical analysis was carried out using SPSS 15.0 statistical software (Chicago, IL). A comparison between Sprague-Dawley groups was performed using two-way ANOVA in conjunction with LSD post-hoc analysis. Statistical analysis for Zucker Obese and Lean groups was conducted using a one-way ANOVA in conjunction with LSD post-hoc analysis. For plasma nitrite results in Study 2, statistical analysis was carried out *via* a paired *t*-test. For all tests, $p \leq 0.05$ was considered significant.

Chapter 3

Study 1: The Effect of Low Vitamin B_6 on Post-initiation Stages of Colon Carcinogenesis

3.1 Study Background and Aims

Epidemiological and experimental studies have implicated vitamin B_6 in reducing the risk of colon cancer. Limited *in vivo* evidence is available making it is unclear if a low B_6 intake increases risk or if a high intake decreases risk.

In this study, we wanted to ensure that we investigate the effect of low B_6 status on colon cancer. High protein intake is a popular dieting trend, particularly by those wanting to increase lean muscle mass. However, due to the interrelationships between protein and vitamin B_6; high dietary protein increases the requirement for vitamin B_6 resulting in lowered circulating levels. Theoretically, this should translate into a reduced availability of vitamin B_6 to the colon, leading one to ask - will high dietary protein compromise vitamin B_6 status and cause an increase in sensitivity to colon carcinogenesis? To answer this question, a study was designed in Sprague-Dawley rats which consists of two different protein levels (normal and high), each containing three different levels of vitamin B_6 (low, normal and high). The rational for including a high-protein group in combination with low B_6 was to determine if vitamin B_6 status was compromised and the implications. Because one can argue that protein itself can be an important nutrient affecting carcinogenesis, a 2x3 factorial design was constructed to distinguish the effect of vitamin B_6 from that of protein.

We also reasoned that if vitamin B_6 functions as an antioxidant, then a reduction in the vitamer should result in increased levels of reactive oxygen species. It is thought that higher levels of reactive oxygen species will enhance pathogenesis resulting in an increased number and growth of putative preneoplastic lesions, ACF. Conversely, supplementary vitamin B_6 is thought to improve redox balance by scavenging reactive

oxygen species and increasing the production of GSH. Reduced ROS and RNS species should decrease DNA damage causing carcinogenesis and thereby reduce ACF growth.

The specific aims of this study were:

- To examine the effect of different levels of vitamin B_6 associated with a normal and high-protein diet during post-initiation stages of colon carcinogenesis in Sprague-Dawley rats on the number and growth of preneoplastic lesions.
- To determine if vitamin B_6 is functioning as an antioxidant at the tissue level.

3.2 Methodological Approach

Details of the experimental procedures are described in Chapter 2, Materials and Methods. Briefly, a 2X3 factorial experimental design was used to determine if three different levels of vitamin B_6, classified as low, normal and high, would affect the physiological level of oxidative stress markers, and the growth and number of ACF, at two different levels of protein intake, classified as normal or high, in Sprague-Dawley rats. See Figure 5 for a detailed illustration of the experimental design. Six week-old male Sprague Dawley rats (n=48) were placed on standard lab chow upon arrival (Rodent Diet 5001, Labdiet, MO, USA). Following a one-week period of acclimation to laboratory conditions, they were subcutaneously injected with the colon-specific carcinogen AOM diluted in 0.9% saline at a dose of 15 mg/kg per body weight, once a week for two weeks. Animals were given free access to standard lab chow and water during the injection period. One week following the second injection, the animals were randomly divided into six groups, each containing eight animals. Each animal group received a different experimental diet for eight weeks that was modified with respect to pyridoxine and casein content (Table 1). Food consumption was measured twice a week. The complete composition of the experimental diets is shown in Appendix B, Table 1.

Body weights of the animals were monitored weekly during dietary treatment and at termination. Animals were terminated after eight weeks of feeding (nine weeks after the last AOM injection). Prior to termination, animals were fasted for 24 hours and sacrificed by CO_2 asphyxiation. After recording body weight, blood was collected. Whole blood

was sent for CBC analysis using rat specific parameters. Plasma samples were analyzed for biochemistry and assessed for levels of homocysteine, cysteine and PLP and oxidative stress markers 3-OH-DG and 3-nitrotyrosine. Weights of heart, liver, spleen and kidney were recorded and samples were stored at -80ºC for future analysis. Any pathologic anomalies observed with the naked eye were recorded as general observations. Colons were fixed and analyzed for growth and development of ACF. Liver tissue was analyzed for levels of oxidative stress markers.

Table 1. Composition of experimental diet groups

Experimental Diet Groups	Protein (Casein)	Vitamin B_6
High Protein, Low B_6	400 g/Kg	3.5 mg/kg
High Protein, Normal B_6	400 g/Kg	7 mg/kg
High Protein, High B_6	400 g/Kg	14 mg/kg
Normal Protein, Low B_6	200 g/Kg	3.5 mg/kg
Normal Protein, Normal B_6	200 g/Kg	7 mg/kg
Normal Protein, High B_6	200 g/Kg	14 mg/kg

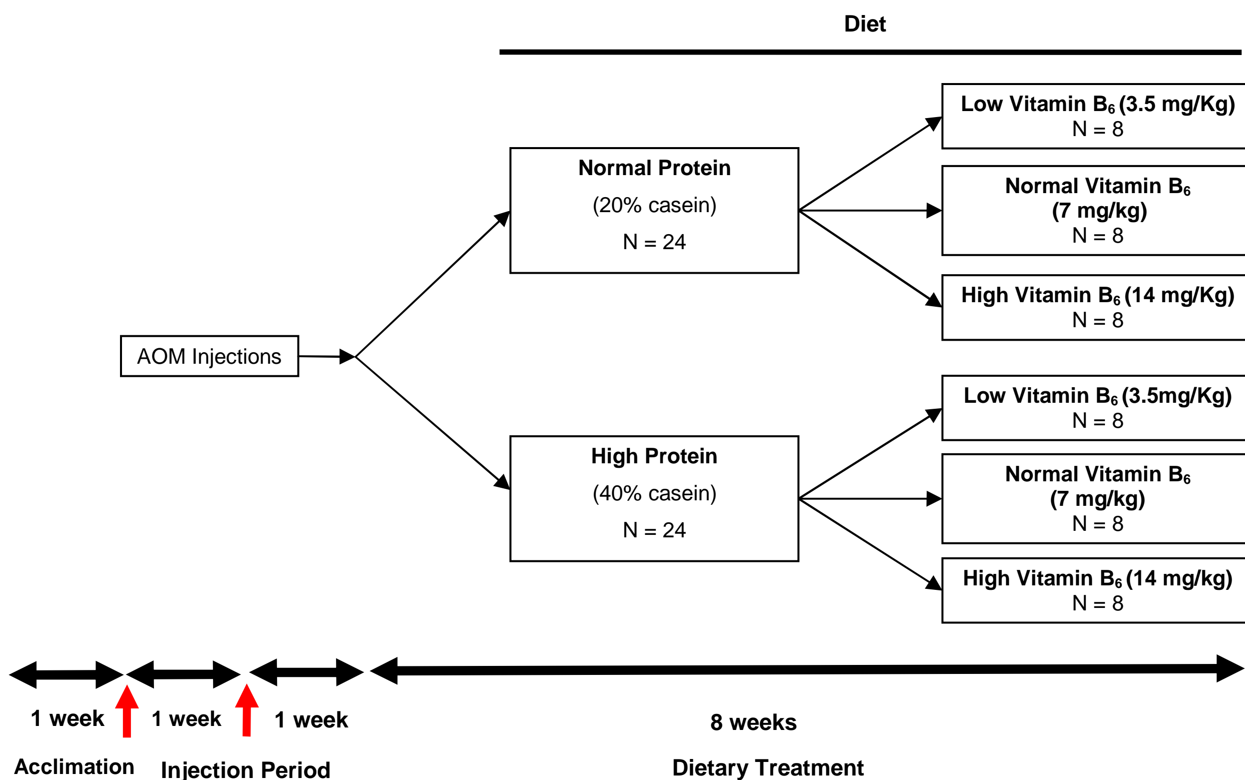

Figure 6. Schematic representation of the experimental protocol for male Sprague-Dawley rats receiving diets varying in protein and vitamin B_6

Red arrows designate AOM injections. Animals were fed standard lab chow during carcinogen injection. One week after the second injection, the animals were randomly divided into six groups and placed on experimental diets. Animals were terminated after eight weeks of feeding (nine weeks after the last AOM injection).

3.3 Results

3.3.1 Diet Consumption and Weekly Body Weights

The amount of diet consumed by each treatment group and the body weights of each rat were monitored weekly. A graph describing the trend of dietary consumption is shown in Figure 6 (for details see Appendix C, Table 2). In week 1, the amount of food consumed was similar between the treatment groups. The amount of food consumed in week 2 decreased approximately 40% across all treatment groups. As dietary treatment continued from week 3 to week 8, the high protein, low B_6 group consumed 100-250 grams more than all other groups. It was noted that the amount of food consumed and body weights of the high protein, low and normal B_6 and normal protein, low B_6 groups were always similar to each other and lower than the remaining three treatment groups. The trend in weekly diet consumption is reflected in the weekly average weight of a rat in each treatment group. The average weekly weight of an animal in each treatment group is listed in Appendix C, Table 1.

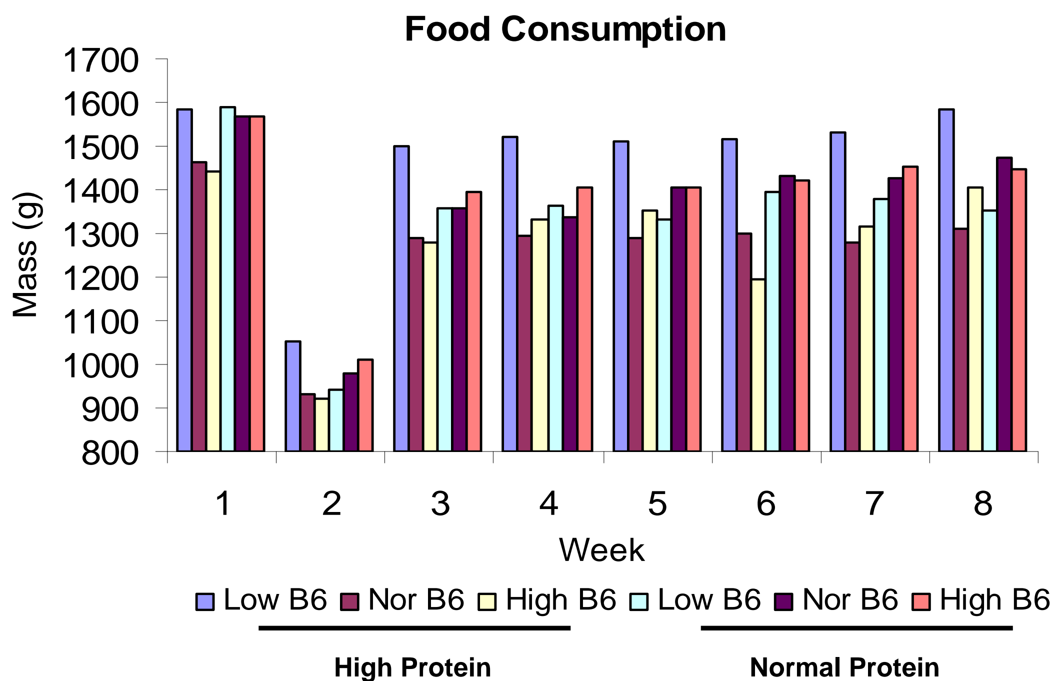

Figure 7. Mass of diet consumed weekly by male AOM-injected Sprague-Dawley rats after eight weeks of intervention with diets varying in protein and vitamin B_6

3.3.2 Final Organ and Body Weights

At termination, the body and organ weights were recorded. Groups of animals receiving high protein diets with normal and high B_6 had significantly lower body weights than groups receiving normal protein with normal or high B_6 (Table 3). In addition, it was noted that the animal group receiving high protein, low vitamin B_6 had a significantly higher body weight than the other two high protein dietary groups. Statistically, a dual effect of protein and vitamin B_6 was observed on the animal groups through body weight.

With the exception of the heart, the organ weights of the high protein, low B_6 animal group were higher than the other groups (Table 3). In particular, the average kidney weight of this group was significantly higher than that of the others. As a group, animals receiving high dietary protein had significantly higher kidney weights than the kidney weights of the groups receiving normal protein. And while not significantly higher, the average liver weight of the high protein, low B_6 animal also appeared noticeably higher than that of the other groups.

Table 2. Main effect of dietary protein and vitamin B_6 on final body and organ weights in AOM-injected male Sprague-Dawley rats after eight weeks of treatment[a].

Variable	Protein Effect	Vitamin B_6 Effect	B_6*Protein Interaction
Body Weight	P = 0.085	P = 0.760	P = 0.031
Heart	P = 0.822	P = 0.845	P = 0.141
Liver	P = 0.908	P = 0.278	P = 0.021
Kidney	P = 0.007	P = 0.165	P = 0.194
Spleen	P = 0.852	P = 0.318	P = 0.098

[a]Effect is significant if $P \leq 0.05$. Statistical analysis is based on a two-way ANOVA with LSD post hoc tests.

Table 3. Final body and organ weights of AOM-injected male Sprague-Dawley rats after eight weeks of diet intervention including changes to dietary protein and vitamin B_6[a]

Group	Body Weight (g)	Heart Weight (g)	Liver Weight (g)	Kidney Weight (g)	Spleen Weight (g)
High Protein, Low B_6	550.0 ± 14.9^{x}	4.2 ± 0.1^{x}	17.5 ± 0.9^{x}	4.1 ± 0.1^{x}	1.0 ± 0.1^{x}
High Protein, Normal B_6	498.6 ± 11.6^{y}	3.8 ± 0.2^{x}	13.9 ± 0.5^{y}	3.7 ± 0.1^{w}	0.9 ± 0.05^{xy}
High Protein, High B_6	501.6 ± 18.5^{y}	3.8 ± 0.2^{x}	15.4 ± 0.7^{xy}	4.0 ± 0.1^{xy}	0.85 ± 0.03^{y}
Normal Protein, Low B_6	521.2 ± 20.1^{xy}	3.8 ± 0.1^{x}	14.9 ± 0.9^{y}	3.6 ± 0.1^{wy}	0.9 ± 0.06^{xy}
Normal Protein, Normal B_6	549.0 ± 19.1^{x}	4.0 ± 0.1^{x}	15.9 ± 0.9^{xy}	3.6 ± 0.2^{wy}	0.90 ± 0.05^{xy}
Normal Protein, High B_6	551.5 ± 13.4^{x}	4.0 ± 0.2^{x}	15.8 ± 0.9^{xy}	3.7 ± 0.2^{wy}	1.00 ± 0.05^{xy}

[a]Values are mean ± SE of eight animals. Means within a column not sharing a common superscript (x, y, z) are significantly different ($P \leq 0.05$, ANOVA analyses with LSD post hoc tests)

3.3.3 Hematological Status

Following termination, whole blood of Sprague-Dawley rats was analyzed for complete blood count (Appendix C, Table 3). Dietary protein exerted effects on a selected number of hematological parameters. Platelet count as well as monocyte count, were significantly lower in rats receiving a high protein diet ($P \leq 0.05$). Analysis of lymphocytes indicated that vitamin B_6 was having an effect. Lymphocyte counts in groups receiving high vitamin B_6 treatment were lower than in the groups receiving low ($P=0.107$) and normal B_6 ($P=0.018$).

3.3.4 Clinical Status

The effect varying dosages of protein and vitamin B_6 has on plasma biochemistry was measured and compared (Appendix C, Table 4). One specific trend observed was the effect of increased dietary protein on a number of biochemical parameters. As a group, high protein significantly increased plasma urea and AST compared to normal protein (Table 6). The high-protein group also had significantly lower HDL-C and cholesterol compared to those that received a normal level of protein ($P \leq 0.05$). Yet, a comparison by individual treatment groups indicates that the level of HDL-C and cholesterol in plasma of the high protein, low B_6 group was comparable to that in the normal protein, low B_6 group. Also, the high protein group had significantly lower triglycerides when compared to the normal protein group. Significant differences in glucose levels were not observed between the individual treatment groups.

Table 4. Complete blood count analyses for AOM-injected male Sprague-Dawley rats after eight weeks of intervention with diets varying in protein and vitamin B_6[a]

	High Protein			Normal Protein		
Parameters	Low B_6	Normal B_6	High B_6	Low B_6	Normal B_6	High B_6
Platelet (10^9/L)	884.0±65.0^{xy}	883.3±25.6^{xy}	866.5±38.9^{x}	935.3±38.0^{xy}	1000.3±41.4^{y}	953.1±48.6^{xy}
Lymphocyte Count (10^9/L)	10.3±0.9^{x}	11.2±1.1^{x}	7.3±1.0^{y}	10.1±1.3^{xy}	10.9±1.0^{x}	9.8±0.6^{y}
Monocyte Count (10^9/L)	0.29±0.04^{xy}	0.21±0.02^{xy}	0.24±0.06^{x}	0.31±0.05^{x}	0.28±0.05^{xy}	0.40±0.05^{y}

[a]Values are mean ± SE of eight animals. Means within a row not sharing a common superscript (x, y, z) are significantly different (P≤0.05, ANOVA analyses with LSD post hoc tests).

Table 5. Main effect of dietary protein and vitamin B_6 on hematological markers in AOM-injected male Sprague-Dawley rats after eight weeks of treatment[a].

Hematological Marker	Protein Effect	Vitamin B_6 Effect	Protein* B_6 Interaction
Platelet	P = 0.024	P = 0.711	P = 0.763
Lymphocyte Count	P = 0.438	P = 0.052	P = 0.295
Monocyte Count	P = 0.020	P = 0.275	P = 0.292

[a]Effect is significant if P≤0.05. Statistical analysis is based on a two-way ANOVA with LSD post hoc tests.

Table 6. Clinical status of AOM-injected male Sprague-Dawley rats after eight weeks of intervention with diets varying in protein and vitamin B_6[a]

	High Protein			Normal Protein		
	Low B_6	Normal B_6	High B_6	Low B_6	Normal B_6	High B_6
Urea (mmol/L)	5.9±0.2^{x}	5.6±0.2^{x}	5.1±0.3^{xy}	4.6±0.3^{y}	5.1±0.4^{xy}	5.1±0.3^{xy}
AST (U/L)	103.3±8.2^{xz}	99.6±5.1^{xz}	109.0±4.2^{x}	79.4±5.3^{y}	92.8±5.7^{z}	80.8±4.0^{y}
HDL-C (mmol/L)	1.8±0.1^{xy}	1.5±0.1^{x}	1.6±0.2^{x}	1.9±0.2^{xy}	2.0±0.2^{y}	2.0±0.2^{y}
Cholesterol (mmol/L)	2.2±0.2^{xy}	1.8±0.1^{x}	1.9±0.2^{x}	2.2±0.2^{xy}	2.4±0.2^{y}	2.4±0.2^{y}
Triglycerides (mmol/L)	1.4±0.4^{x}	0.8±0.1^{y}	0.8±0.1^{y}	1.4±0.3^{x}	1.2±0.1^{x}	1.7±0.2^{x}

[a]Values are mean ± SE of eight animals. Means within a row not sharing a common superscript (x, y, z) are significantly different (P≤0.05, two-way ANOVA analyses with LSD post hoc tests). Abbreviations: AST, aspartate aminotransferase; HDL-C, High-density lipoproteins.

Table 7. Main effect of dietary protein and vitamin B_6 on clinical status in AOM-injected male Sprague-Dawley rats after eight weeks of treatment[a].

Biochemistry Marker	**Protein Effect**	**Vitamin B_6 Effect**	**B_6*Protein Interaction**
Urea	P = 0.020	P = 0.740	P = 0.120
AST	P = 0.000	P = 0.666	P = 0.141
HDL-C	P = 0.005	P = 0.952	P = 0.308
Cholesterol	P = 0.010	P = 0.828	P = 0.205
Triglycerides	P = 0.019	P = 0.181	P = 0.195

[a]Effect is significant if P≤0.05. Statistical analysis is based on a two-way ANOVA with LSD post hoc tests.

3.3.5 Levels of Hepatic Cholesterol

To compare the concentration of cholesterol in hepatic tissue of the Sprague-Dawley treatment groups, an equal amount of protein, 50 µg, was loaded for each sample. Six samples of liver tissue from each treatment group were randomly selected for the cholesterol assay. The results of the liver cholesterol assay are illustrated in Figure 7. Analysis of plasma cholesterol indicated a significant decrease in levels by high protein dietary treatment. While not significant, a similar trend was observed in the analysis of liver cholesterol; Sprague-Dawley rats receiving high dietary protein had lower hepatic cholesterol ($P=0.086$). Furthermore, regardless of the protein level, treatment groups receiving high vitamin B_6 had higher levels of liver cholesterol than normal B_6 groups.

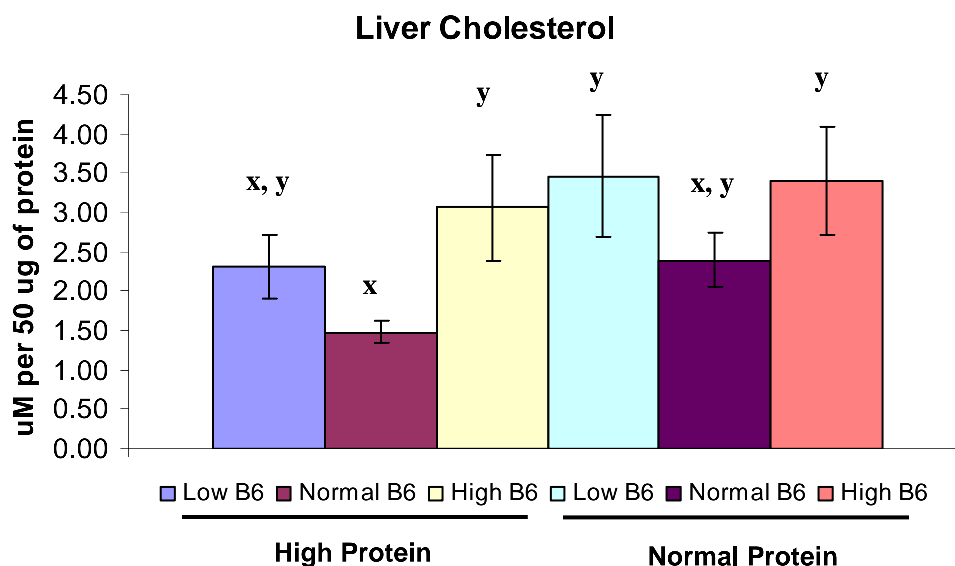

Figure 8. Liver cholesterol levels in AOM-injected male Sprague-Dawley rats after eight weeks of intervention with diets varying in protein and vitamin B_6.

Values are expressed as mean ± SE, N=6 per group. Bars not sharing a common superscript (x, y) are significantly different (P≤0.05, ANOVA analyses with LSD post hoc tests).

Table 8. Main effect of dietary protein and vitamin B_6 on hepatic cholesterol in AOM-injected male Sprague-Dawley rats after eight weeks of treatment[a].

	Protein Effect	Vitamin B_6 Effect	B_6*Protein Interaction
Cholesterol	P = 0.086	P = 0.069	P = 0.752

[a]Effect is significant if P≤0.05. Statistical analysis is based on a two-way ANOVA with LSD post hoc tests.

3.3.6 Enumeration of Aberrant Crypt Foci (ACF)

After eight weeks of feeding the animals with the experimental diets, the animal's colons were examined for preneoplastic changes and compared to a group of AOM-injected rats receiving normal levels of dietary protein and vitamin B_6. An interesting trend was observed - the high protein, low B_6 animal group had significantly lower total and primal ACF ($P \leq 0.05$) (Table 10). However, the average number of total crypts, intermediate and advanced ACF for the normal protein, high B_6 group behaved similarly to the other groups with the exception of the high protein, low B_6 group. The total ACF count and primal, intermediate and advanced classification for the high protein groups with normal and high B_6 behaved similarly to the normal protein groups with low and normal B_6.

Table 9. Main effect of dietary protein and vitamin B_6 on ACF growth in AOM-injected male Sprague-Dawley rats after eight weeks of treatment[a].

	Protein	Vitamin B_6	Protein*Vitamin B_6 Interaction
Total ACF	P = 0.145	P = 0.132	P = 0.255
Primal ACF	P = 0.174	P = 0.135	P = 0.176
Intermediate ACF	P = 0.320	P = 0.756	P = 0.390
Advanced ACF	P = 0.718	P = 0.487	P = 0.421

[a]Effect is significant if $P \leq 0.05$. Statistical analysis is based on a two-way ANOVA with LSD post hoc tests.

Table 10. Enumeration of ACF growth characteristics[1,2] in AOM-injected male Sprague-Dawley rats after eight weeks of intervention with diets varying in protein and vitamin B_6

Diet Group	Total ACF	Primal ACF (1-3 Crypts)	Advanced ACF (4-6 Crypts)	Intermediate ACF (≥ 7 Crypts)
High Protein, Low B_6	210.1 ± 22.8^x	190.4±17.8^x	19.0±5.3^x	0.7±0.4^x
High Protein, Normal B_6	301.6 ± 31.0^y	281.1±28.7^y	19.8±3.3^x	0.8±0.5^x
High Protein, High B_6	320.0 ± 15.9^y	301.8±17.9^y	17.1±2.9^x	1.1±0.4^x
Normal Protein, Low B_6	309.0 ± 33.7^y	289.1±31.8^y	20.4±3.7^x	0.4±0.2^x
Normal Protein, Normal B_6	313.0 ± 24.7^y	289.9±23.5^y	18.8±3.6^x	1.1±0.6^x
Normal Protein, High B_6	323.3 ± 46.9^y	294.8±23.5^y	27.3±6.1^x	1.3±0.6^x

1. Values are expressed as mean ± SE. N=7 for high protein, low B_6 group, and N=8 all other groups.
2. Values in the same column not sharing a common superscript are significantly different (P≤0.05, ANOVA analyses with LSD post hoc tests)

3.3.7 Plasma Markers of the Transulfuration Pathway

Plasma levels of pyridoxal-5'-phosphate (PLP), the active form of vitamin B_6, were measured by HPLC analysis as described previously. Five plasma samples from each group were randomly selected for analysis. Sprague-Dawley rats receiving normal protein, high vitamin B_6 treatment had significantly higher levels of plasma PLP ($P \leq 0.05$) (Table 11). The level of plasma PLP in the high protein, low B_6 group was comparable to the two other high protein groups and the normal protein groups containing low and normal vitamin B_6.

The levels of plasma homocysteine and cysteine did not change in a consistent manner; the results are summarized in Table 11. Five plasma samples from each group were analyzed. Compared to the groups receiving normal dietary protein, the high protein groups had reduced plasma homocysteine levels ($p=0.101$). The level of plasma cysteine in the normal protein, high B_6 group was lower than the high protein groups receiving low and high B_6 ($p \leq 0.05$). The normal protein, low B_6 group also had higher plasma cysteine than the normal protein, high B_6 group ($p \leq 0.05$). With the exception of the high protein, normal B_6 group, it was observed that vitamin B_6 was lowering cysteine levels with increased dosage ($P=0.092$).

The level of GSH in plasma was assayed by colorimetric determination as previously described. Dietary treatment of vitamin B_6 was observed to exert an effect on the level of plasma glutathione ($p=0.079$). Sprague-Dawley rats receiving low vitamin B_6 had lower levels of GSH than normal ($P=0.030$) and high B_6 ($P=0.074$) treatment groups. The highest levels of reduced GSH in plasma were measured in the normal protein groups receiving normal and high B_6.

Table 11. Plasma pyridoxal 5'-phosphate, homocysteine, cysteine and glutathione levels[1,2,3] in AOM-injected male Sprague-Dawley rats after eight weeks of intervention with diets varying in protein and vitamin B_6

	High Protein			Normal Protein		
	Low B_6	Normal B_6	High B_6	Low B_6	Normal B_6	High B_6
PLP (nmoles/L)	985.3±130.1^{x}	1063.6±191.8^{x}	955.9±115.8^{x}	974.4±113.2^{x}	988.5±80.3^{x}	1577.4±210.0^{y}
Homocysteine (µM)	5.9 ± 0.7^{xy}	6.0 ± 0.3^{xy}	5.3 ± 0.4^{x}	6.5 ± 0.9^{xy}	7.2 ± 0.8^{y}	6.2 ± 0.8^{xy}
Cysteine (µM)	249.5±6.6^{x}	208.9±13.0^{y}	257.2±8.1^{x}	244.7±17.9^{xy}	226.6±16.1^{xy}	210.1±10.9^{y}
GSH (µM)	314.8±53.5^{xy}	332.5±45.5^{xy}	345.7±5.3^{xy}	270.1±26.3^{y}	401.6±15.0^{x}	362.9±34.6^{x}

1. Values are expressed as mean ± SE. N=5 for all dietary treatment groups. N=5 for all assays.
2. Values in the same row not sharing a common superscript (x, y, z) are significantly different (P≤0.05, ANOVA analyses with LSD post hoc tests).
3. Abbreviations are as follows: PLP, Pyridoxal 5'-Phosphate; GSH, glutathione.

Table 12. Main effect of dietary protein and vitamin B_6 on plasma PLP, homocysteine, cysteine and GSH in AOM-injected male Sprague-Dawley rats after eight weeks of treatment[a].

Variable	Protein Effect	Vitamin B_6 Effect	Protein* B_6 Interaction
PLP	P = 0.151	P = 0.135	P= 0.050
Homocysteine	P = 0.101	P = 0.447	P = 0.912
Cysteine	P = 0.286	P = 0.092	P = 0.053
GSH	P = 0.618	P = 0.079	P = 0.264

[a]Effect is significant if P≤0.05. Statistical analysis is based on a two-way ANOVA with LSD post hoc tests.

3.3.8 Oxidative Stress Assessment

Vitamin B_6 altered GSH levels in hepatic tissue ($P \leq 0.05$) (Figure 8). With increasing levels of vitamin B_6, the level of liver GSH increased. The high protein, normal B_6 group had significantly higher GSH level than the normal protein, low B_6 group ($P \leq 0.05$). Significant differences were not observed in hepatic NO_2^- (Figure 9) and in the activities of antioxidant enzymes SOD and CAT (Table 14).

Sprague-Dawley plasma was also analyzed for 8-hydroxy-deoxyguanosine (8-OH-DG) and 3-nitrotyrosine. Since 8-OH-DG is a representative ROS biomarker and nitrotyrosine is a marker for RNS, we were able to gain further insight into the affect of dietary treatment on the redox balance within the plasma. Five plasma samples from each dietary treatment group were selected for both analyses. Dietary protein had a significant effect on the levels of plasma 8-OH-DG ($P \leq 0.05$) (Figure 10). Results from 8-OH-DG analysis in plasma demonstrate a stepwise increase in the levels of the biomarker moving from the high protein, low B_6 group to the normal protein, high B_6 group. The level of dietary protein also had a significant affect on hepatic nitrotyrosine concentrations ($P \leq 0.05$) (Figure 11). Animals receiving high protein had lower levels of liver nitrotyrosine than normal protein groups. The concentrations of nitro-tyrosine within each protein subgroup did not vary significantly.

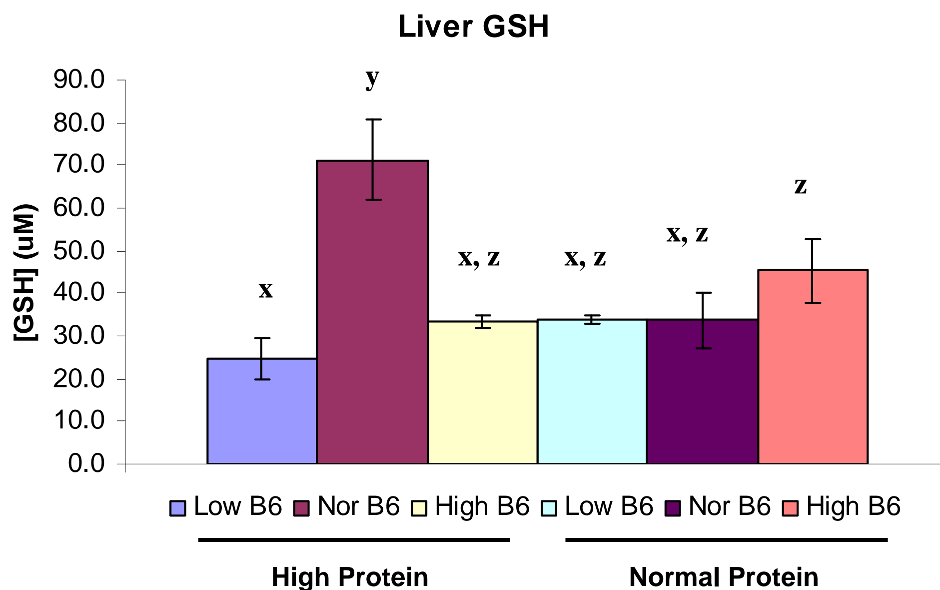

Figure 9. Hepatic glutathione levels in AOM-injected male Sprague-Dawley rats after eight weeks of intervention in dietary protein and vitamin B_6

Values are expressed as mean ± SE, N=6 per dietary group. Bars not sharing a common superscript (x, y, z) are significantly different ($P \leq 0.05$, ANOVA analyses with LSD post hoc tests). Abbreviation: GSH, glutathione.

Table 13. Main effect of dietary protein and vitamin B_6 on hepatic GSH and NO_2^- in AOM-injected male Sprague-Dawley rats after eight weeks of treatment[a].

	Protein Effect	Vitamin B_6 Effect	Protein*Vitamin B_6 Interaction
GSH	P = 0.270	P = 0.030	P = 0.000
NO_2^-	P = 0.607	P = 0.637	P = 0.257

[a]Effect is significant if $P \leq 0.05$. Statistical analysis is based on a two-way ANOVA with LSD post hoc tests.

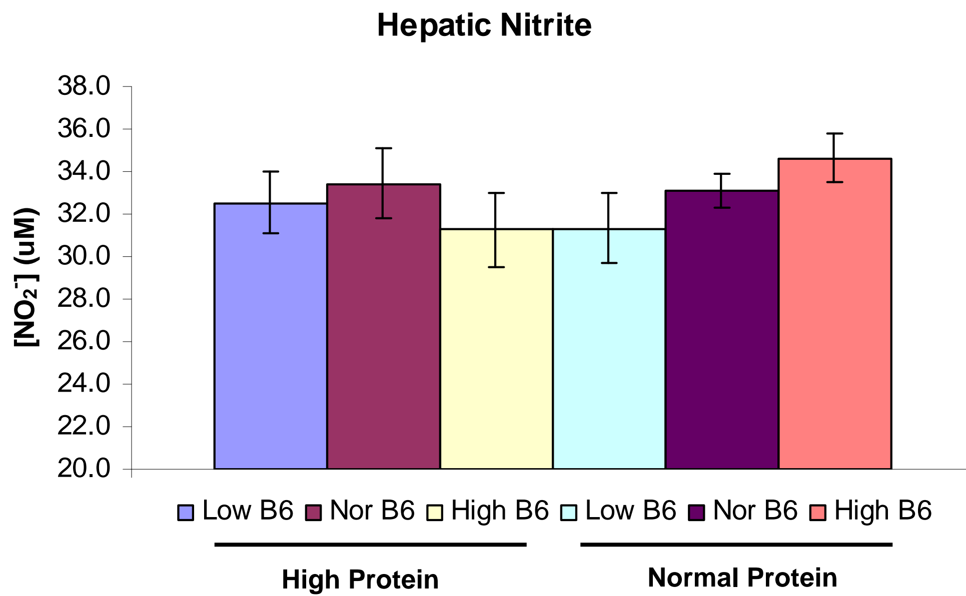

Figure 10. Hepatic nitrite levels in AOM-Injected male Sprague-Dawley rats after eight weeks of intervention with diets varying in protein and vitamin B_6

Values are expressed as mean ± SE, N=7 per dietary group. Abbreviation: NO_2^-, nitrite.

Table 14. Superoxide dismutase and catalase activities[1,2,3,4,5] in liver tissue of AOM-injected male Sprague-Dawley rats after eight weeks of intervention with diets varying in protein and vitamin B_6

	High Protein			Normal Protein		
	Low B_6	Normal B_6	High B_6	Low B_6	Normal B_6	High B_6
SOD (U/mg)	0.09 ± 0.02^{x}	0.11 ± 0.02^{xy}	0.06 ± 0.01^{x}	0.06 ± 0.02^{x}	0.16 ± 0.03^{y}	0.09 ± 0.01^{x}
CAT (U/mg)	1434.8±199.8	1301.9±76.8	1257.3±196.0	1445.0±80.0	1337.4±102.3	1276.2±214.4

1. Values are expressed as mean ± SE, N=6 for all treatment groups.
2. Values in the same row not sharing a common superscript (x, y) are significantly different (P≤0.05, ANOVA analyses with LSD post hoc tests).
3. For SOD activity, one unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.
4. For CAT activity, one unit is defined as the amount of enzyme that will cause formation of 1.0 nmol formaldehyde per minute at 25°C.
5. Abbreviations: SOD, Superoxide Dismutase activity; CAT, Catalase activity.

Table 15. Main effect of dietary protein and vitamin B_6 on hepatic SOD and CAT activities in AOM-injected male Sprague-Dawley rats after eight weeks of treatment[a].

Antioxidant Enzyme	Protein Effect	Vitamin B_6 Effect	Protein*Vitamin B_6 Interaction
SOD	P = 0.369	P = 0.006	P = 0.118
CAT	P = 0.867	P = 0.533	P = 0.997

[a]Effect is significant if P≤0.05. Statistical analysis is based on a two-way ANOVA with LSD post hoc tests.

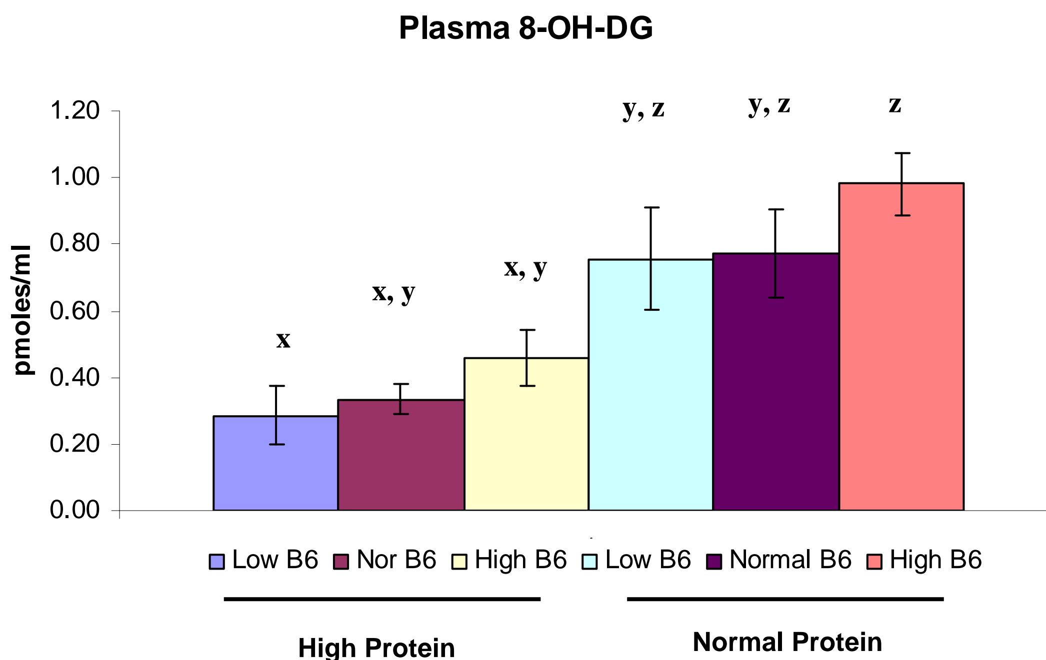

Figure 11. Level of plasma 8-OH-DG in AOM-injected male Sprague-Dawley rats after eight weeks of intervention with diets varying in protein and vitamin B_6

Values are expressed as mean ± SE, N=5 for all dietary treatment groups. Bars not sharing a common superscript are significantly different ($P \leq 0.05$, ANOVA analysis with LSD post hoc tests). Abbreviations: 8-OH-DG, 8-hydroxy-2-deoxyguanosine; AOM, azoxymethane.

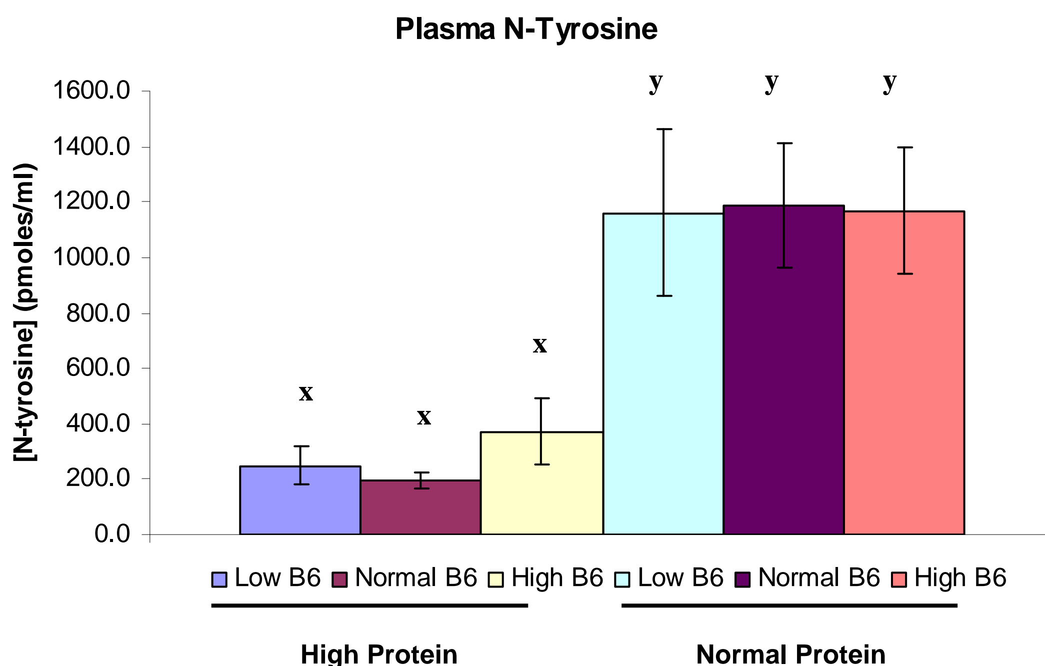

Figure 12. Level of plasma 3-nitrotyrosine in AOM-injected male Sprague-Dawley rats after eight weeks of intervention with diets varying in protein and vitamin B_6

Values are expressed as mean ± SE, N=5 for all dietary treatment groups. Bars not sharing a common superscript are significantly different (P≤0.05, ANOVA analysis with LSD post hoc tests).

Table 16. Main effect of dietary protein and vitamin B_6 on plasma 8-OH-DG and N-tyrosine in AOM-injected male Sprague-Dawley rats after eight weeks of treatment[a].

Oxidative Stress Marker	Protein Effect	Vitamin B_6 Effect	Protein*Vitamin B_6 Interaction
8-OH-DG	P = 0.000	P = 0.149	P = 0.921
N-Tyrosine	P = 0.000	P = 0.906	P = 0.874

[a]Effect is significant if P≤0.05. Statistical analysis is based on a two-way ANOVA with LSD post hoc tests.

3.4 Discussion

The study was designed to determine if a high protein diet will compromise the vitamin B_6 status which, in turn, will affect the measurements reflecting impaired amino acid metabolism, an increase in oxidative stress markers and growth of ACF. It was noted that the group fed high protein in combination with low B_6 had the lowest number of ACF. It became apparent that the level of protein was an important variable in altering a number of physiological parameters. The plasma level of cholesterol, platelet and monocytes counts, and the oxidative stress markers were lower in high protein than in normal protein groups. The level of B_6 was selected based on the normal recommended level in the rodent diet. It is possible that the recommended level, with a safety factor, is sufficient even if one increases the amount of protein in the diet, as was done in the present study. Since ACF number was lower in the high protein, low B_6 group, it can be suggested that preneoplastic lesions also require adequate vitamin B_6 for their growth. The normal protein diet contained higher carbohydrates than the high protein diet. Oxidative stress markers were higher in the normal protein group than the high protein group. Again, these differences could have been due to high carbohydrates and rising glucose burden and metabolism, leading to a resulting increase RNS and ROS production.

As expected, Sprague-Dawley rats receiving a supplementary amount of pyridoxine at 14 mg/kg (normal protein, high B_6 group) had the highest concentration of plasma PLP. Previous research reports an inverse correlation between the concentrations of circulating vitamin B_6 compounds (urinary 4-pyridoxic acid and plasma pyridoxal 5'-phosphate) and protein intake (Miller et al. 1985). It has been suggested that as the level of dietary protein is increased, more vitamin B_6 is removed from circulation and retained in the liver to supply PLP to PLP-dependent enzymes involved in the catabolism of excess amino acids. As a result, less vitamin B_6 is available for conversion to 4-PA and PLP (Miller et al., 1985 and Lumeng et al., 1974). Unlike previous studies, results of plasma PLP analyses from high protein groups in this study did not follow this trend. Plasma PLP levels in the high protein group did not vary significantly from the normal protein, low and normal B_6 groups. With the high protein, high B_6 group specifically, it is plausible that the amount of pyridoxine treatment was sufficient to balance out the requirements of PLP-dependent enzymes and

maintain redox balance despite increases in protein. Yet, the plasma PLP levels in the high protein, low and normal B_6 groups were contrary to our hypothesis. There is some evidence to suggest that, in rats, plasma PLP levels may not accurately reflect vitamin B_6 status (Schaeffer et. al., 1989). We also know that protein metabolism was increased in the livers of high protein, normal and low B_6 groups as evidenced by plasma urea concentrations. The metabolism of proteins and amino acids in these groups resulted in an increase in deamination leading to urea as the final degradation product for excretion of excess nitrogen. This corresponds to previous research that indicates an increase in urea synthesis during high dietary protein intake and would suggest a greater requirement for PLP as coenzyme for protein metabolism in the liver of these groups (Saheki et. al., 1978). Therefore, before concluding that a low and normal B_6 dose is adequate during high protein intake, additional experiments evaluating vitamin B_6 status would be recommended. Experiments could include measuring urinary 4-PA excretion, erythrocyte alanine or aspartate aminotransferase stimulation and microbiological assessment in hepatic tissue using *Saccharomyces uvarum*.

No definite trend was observed for plasma PLP, homocysteine and cysteine in the treatment groups. The metabolism of homocysteine to cysteine requires PLP as cofactor for two enzymes in the transulfuration pathway. By supplementing pyridoxine, a decrease in plasma homocysteine and an increase in cysteine were expected. From the results, one can conclude that vitamin B_6 at different levels of protein did not affect this pathway.

GSH is an important antioxidant and the cellular concentration of this metabolite has a major effect on antioxidant function. There was no consistent affect of dietary treatment on plasma and liver GSH.

Vitamin B_6 had no effect on plasma 3-nitrotyrosine. Rather, protein intake affected levels of 3-nitrotyrosine. High protein had lower levels of 3-nitrotyrosine compared to normal protein. 3-Nitrotyrosine is marker used to detect the presence of peroxynitrite ($ONOO^-$) and is produced from a reaction between $ONOO^-$ and L-tyrosine (Maruyama et. al., 1996). The formation of ROS and RNS during oxidative stress can be assessed by their reaction products. Nitric oxide (NO) and superoxide anion may react together to produce $ONOO^-$. Peroxynitrite is a more active molecule and a powerful oxidizing agent that can initiate

nitration and hydroxylation of aromatic rings (Kumarathasan and Vincent, 2003). Peroxynitrite can cause DNA fragmentation and lipid oxidation. Since 3-nitrotyrosine is a biomarker $ONOO^-$ and groups receiving high protein had lower levels of the nitrated aromatic ring, one can suggest that high protein or low carbohydrate diet lead to less oxidative damage than normal protein counterparts.

In addition to forming peroxynitrite, NO is also oxidized to nitrite and nitrate. Since NO_2^- also serves as a marker of NO, a trend similar to 3-nitrotyrosine levels was expected. The level of liver NO_2^- did not vary significantly among treatment groups. This could be attributed to the sensitivity of the techniques used. The high performance liquid chromatography method used to detect nitro-tyrosine has been documented to be a more sensitive assay than the one used to calorimetrically determine nitrite concentration. Another factor that might have affected colorimetric determination of nitrite was interferences due to antioxidants and nucleophiles (i.e. GSH and cysteine).

The most pro-mutagenic oxidation product of guanine is 8-hydroxy-2-deoxyguanosine (8-OH-DG) (Thompson, 2006). It is easily formed and is a potential biomarker for carcinogenesis as it can give rise to G-to-T transversion mutations in several genes (Thompson, 2006). A stepwise trend was observed for plasma 8-OH-DG results. By increasing dietary protein and decreasing B_6 intake, the production of 8-OH-DG was decreased. In early carcinogenesis, the high protein diet in combination with low B_6 had the lowest concentration of plasma 8-OH-DG and ACF count. One can infer from this result that sensitivity to ACF development could be decreased by increasing protein or lowering carbohydrate, while maintaining adequate B_6 intake.

In conclusion, different levels of vitamin B_6 did not affect early stages of carcinogenesis and did not have a significant effect on the antioxidant status. Contrary to the hypothesis, low vitamin B_6 in combination with high protein reduced the growth and development of ACF. This study also demonstrated that protein and/or carbohydrate level is an important determinant in modulating oxidative stress.

Chapter 4

Study 2: The Role of Supplemental Vitamin B_6 as a Biochemical Response Modifier of Colon Carcinogenesis in Obesity

4.1 Study Background and Specific Aims

The ability of vitamin B_6 to exert a noticeably protective effect and lower oxidative stress could be dependent on the concentration of pyridoxine supplemented and the health of the subject. Humans and experimental animal models diagnosed as Type 1 and Type 2 diabetics have been shown to exhibit vitamin B_6 deficiency (Davis et. al., 1976 and Nair et. al., 1998). Hyperglycemia, a hallmark of diabetes, is known to generate reactive oxygen species (Brownlee, 2001). *In vitro*, vitamin B_6 supplementation has been shown to inhibit superoxide radicals, lipid peroxidation and protein glycosylation in human erythrocytes treated with high glucose (Jain and Lim, 2000). Supplementation with vitamin B_6 has also been shown to lower blood glucose in streptozotocin-treated diabetic animals and glycosylated hemoglobin levels in Type 2 diabetic patients (Nair et. al., 1998). To date there is no experimental evidence documenting the effect of B_6 supplementation on oxidative stress in diabetic patients and animals (Jain, 2007).

Zucker Obese rats are hyperglycemic and at risk of developing Type 2 diabetes. High concentrations of circulating glucose are a symptom of hyperphagia caused by the inheritance of non-functional leptin receptors of the Zucker animal model (Bray, 1977). As previously mentioned, hyperglycemia generates oxidative stress. Additionally, the Zucker Obese animal model is known for representing a sustained inflammatory state and having an increased sensitivity to colon cancer (Gunter and Leitzmann, 2006). It is postulated that the heightened sensitivity of these animals to colon carcinogenesis is due to obesity associated pro-inflammatory responses and elevated levels of reactive oxygen species.

In this study, it was hypothesized that if B_6 functions as an antioxidant, then supplemental B_6 will alleviate ROS and will reduce sensitivity of the colon to the number and growth of ACF, preneoplastic lesions. In the first study, low or high B_6 (2x higher than recommended intake) did not change the oxidative status of Sprague-Dawley rats. Therefore, it was reasoned that,

possibly, a supraphysiological dose of pyridoxine would serve as an antioxidant. Hence, it was prudent to use an animal model with a pre-existing physiological state identified to be pro-inflammatory and high in oxidative stress.

The specific aims of this study were:

- To assess if supplemental vitamin B_6 lowers progression of colon cancer in Zucker Obese rats in post-initiation stages.
- To measure tissue levels of oxidative stress markers.

4.2 Methodological Approach

A group of AOM-injected Zucker Obese rats receiving a diet high in vitamin B_6 was compared to a treatment group consuming the recommended level of vitamin intake. A detailed summary of the study design is illustrated in Figure 12. Six week old female Zucker Obese (n=16) and Zucker Lean rats (n= 20) arrived from Charles River Canada and were housed in the Animal Facility. During the first week, rats were acclimated to laboratory conditions and given free access to standard lab chow. Following acclimation, animals were placed on experimental diets varying in the level of vitamin B_6 for two weeks (Table 17). One Zucker Obese animal group received high vitamin B_6 (35 mg/kg PN-HCl) while another received the recommended amount (7 mg/kg PN-HCl). Both Zucker lean groups received 7 mg/kg PN-HCl. After two weeks of feeding, animals were subcutaneously injected with AOM, diluted in 0.9% saline at a dose of 10 mg/kg body weight. Animals received a second injection two weeks later. Animals were given free access to the experimental diets during and following the injection period. Body weights were monitored weekly during the entire study and six weeks after the last AOM injection, animals were fasted for 24 hours and terminated by CO_2 asphyxiation. After recording body weight, blood was obtained and sent for CBC, biochemical, homocysteine, cysteine and PLP analysis. Weights of heart, liver, spleen and kidney were recorded and samples were stored at -80ºC for future analysis. Any pathologic anomalies noted by the naked eye were recorded as general observations. Colons were analyzed for the growth and development of ACF and liver tissue was analyzed for oxidative stress markers.

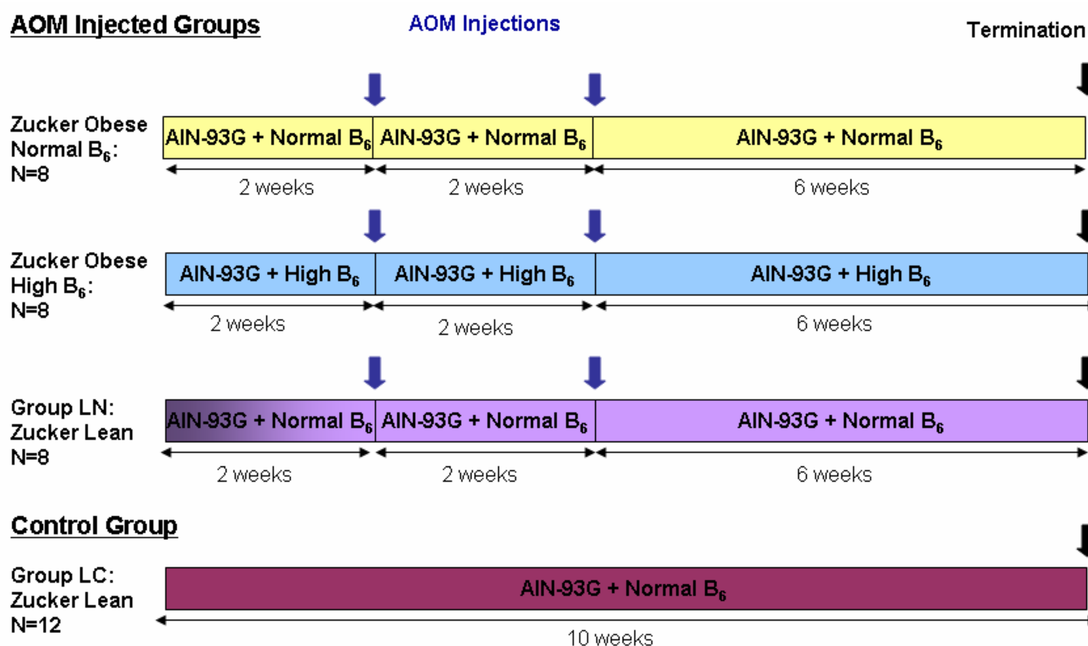

Figure 13. Schematic representation of the experimental protocol for effect of supplementary Vitamin B_6 in the Zucker Obese and Lean animal model.

Blue arrows represent AOM injections and black arrows represent animal termination. Animals were randomly housed in groups of three or four and began feeding on experimental diets two weeks prior to carcinogen injection. Animals were terminated six weeks after the second injection.

Table 17. Amount of vitamin B_6 in experimental diets of Zucker Obese and Lean study

Group	Vitamin B_6 (mg/kg)
Group OH: Zucker Obese, High B_6	35
Group ON: Zucker Obese, Normal B_6	7
Group LN: Zucker Lean, Normal B_6	7
Group LC: Zucker Lean Control, Normal B_6	7

4.3 Results

4.3.1 Body and Organ Weights

At termination, the body and organ weights of the rats were recorded (Table 18). A significant difference between the body weights of Zucker Obese rats receiving normal and high B_6 was not observed. Differences in heart and kidney weights were also not observed between the two obese feeding groups. However, significant differences were observed in the liver and spleen weights of Zucker Obese rats receiving normal B_6 (Zk-OBN) and high B_6 (Zk-OBH) ($p \leq 0.05$). Zucker Obese rats receiving five times the regular does of vitamin B_6 had significantly lower liver weights than those receiving normal levels. During necropsy, the gross appearances of the organs were assessed blindly. The coloration of livers in the Zk-OBH group appeared darker pink and less marble-like in pattern compared to their normal B_6 counterparts. The body and organ weights of Zucker Lean animals were significantly lower than both obese treatment groups ($p \leq 0.05$).

Table 18. Final body and organ weights[1,2,3] of AOM-injected female Zucker Obese and Lean rats after dietary treatment with vitamin B_6

Group	Body Weight (g)	Liver (g)	Kidney (g)	Heart (g)	Spleen (g)
Zk-OBN	550.8 ± 13.5^x	43.8 ± 2.5^x	4.0 ± 0.2^x	1.3 ± 0.0^x	1.1 ± 0.0^x
Zk-OBH	557.2 ± 9.8^x	37.4 ± 2.7^y	3.8 ± 0.2^x	1.3 ± 0.0^x	0.9 ± 0.0^y
Zk-LN	267.0 ± 8.4^y	9.0 ± 2.7^z	2.2 ± 0.1^y	1.0 ± 0.0^y	0.5 ± 0.0^z

1. Values are expressed as mean ± SE, N=8.
2. Values in the same column without a common superscript are significantly different (ANOVA analysis with LSD post hoc tests).
3. Abbreviations are as follows: Zk-OBN, Zucker Obese rats receiving normal levels of Vitamin B_6; Zk-OBH, Zucker Obese rats receiving high Vitamin B_6; Zk-LN, AOM-injected Zucker Lean rats receiving normal vitamin B_6.

4.3.2 Hematological Status

Whole blood from Zucker Obese and Lean rats was analyzed for complete blood count (Appendix C, Table 6). Platelet counts from Zucker Obese rats receiving high B_6 and the Zucker Lean group were significantly lower than the Zucker Obese group receiving normal B_6 ($P \leq 0.05$) (Table 19). Zucker Obese rats receiving high B_6 had significantly lower monocyte counts compared to the Zucker Obese normal B_6 group ($P \leq 0.05$). While not significant, the lymphocyte count of Zucker Obese rats receiving high B_6 was lower than the normal B_6 group ($p=0.098$) and Zucker Lean rats ($p=0.366$).

Table 19. Hematological status[1,2,3] of AOM-injected female Zucker Obese and Lean rats after receiving vitamin B_6 treatment

Parameters	Zk-OBN	Zk-OBH	Zk-LN
Platelets (10^9/L)	975.4 ± 43.3^{x}	833.5 ± 46.8^{y}	800.3 ± 59.4^{y}
Lymphocyte (10^9/L)	4.7 ± 0.7^{x}	3.0 ± 0.6^{x}	3.9 ± 0.9^{x}
Monocyte (10^9/L)	0.4 ± 0.1^{x}	0.3 ± 0.0^{y}	0.1 ± 0.0^{y}

1. Values are expressed as mean ± SE. N=8 for Zk-OBN and Zk-OBH dietary groups and N=6 for Zk-LN.
2. Values in the same row without a common superscript are significantly different ($P<0.05$, ANOVA analyses with LSD post hoc tests).
3. Abbreviations: Zk-OBN, Zucker Obese normal B_6 group; Zk-OBH, Zucker Obese high B_6 group; Zk-LN, AOM-injected Zucker Lean rats receiving normal B_6.

4.3.3 Clinical Assessment

The effect of vitamin B_6 on plasma biochemistry in Zucker Obese and Lean rats was analyzed and compared (Appendix C, Table 7). Total protein was higher in the Zucker Obese group receiving normal B_6 than the high B_6 treatment group (p=0.095) and significantly greater than the Zucker Lean group (P≤0.05) (Table 20). A significant difference in glucose was not observed between the Zucker Obese normal B_6 and high B_6 groups. However, glucose in Zucker Lean rats was significantly lower than the obese groups. Initial analysis of triglyceride values in the Zucker Obese group with N=8 indicated that Zk-OBH had higher levels compared to Zk-OBN and Zk-LN. However, after removal of an outlier that was greater than 3 standard deviations in the Zk-OBH group, a significant difference in the level of plasma triglycerides did not exist between the two obese treatment groups (Figure 13).

Table 20. Clinical assessment[1,2,3] of AOM-injected female Zucker Obese and Lean rats after receiving B_6 treatment

Biochemical Parameters	Zk-OBN	Zk-OBH	Zk-LN
Total Protein (g/L)	80.0 ± 1.7^{x}	76.4 ± 1.4^{x}	75.0 ± 1.3^{y}
Cholesterol (mmol/L)	11.9 ± 0.4^{x}	9.5 ± 1.1^{y}	2.5 ± 0.2^{z}
Glucose (mmol/L)	14.8 ± 0.8^{x}	14.9 ± 1.8^{x}	7.6 ± 0.5^{y}
Triglycerides (mmol/L)	3.5 ± 0.5^{xy}	5.1 ± 1.4^{y}	1.2 ± 0.3^{x}
ALT (U/L)	126.1 ± 35.1^{x}	127.8 ± 19.9^{x}	36.8 ± 1.4^{y}
Globulin (g/L)	37.6 ± 0.8^{x}	33.6 ± 1.4^{y}	22.8 ± 1.3^{z}

1. Values are expressed as mean ± SE. N=8 for Zk-OBN and Zk-OBH dietary groups and N=6 for Zk-LN.
2. Values in the same row without a common superscript are significantly different (P<0.05, ANOVA analysis with LSD post hoc tests).
3. Abbreviations: Zk-OBN, Zucker Obese normal B_6 group; Zk-OBH, Zucker Obese high B_6 group; Zk-LN, AOM-injected Zucker Lean rats receiving normal B_6, ALT, Alanine aminotransferase.

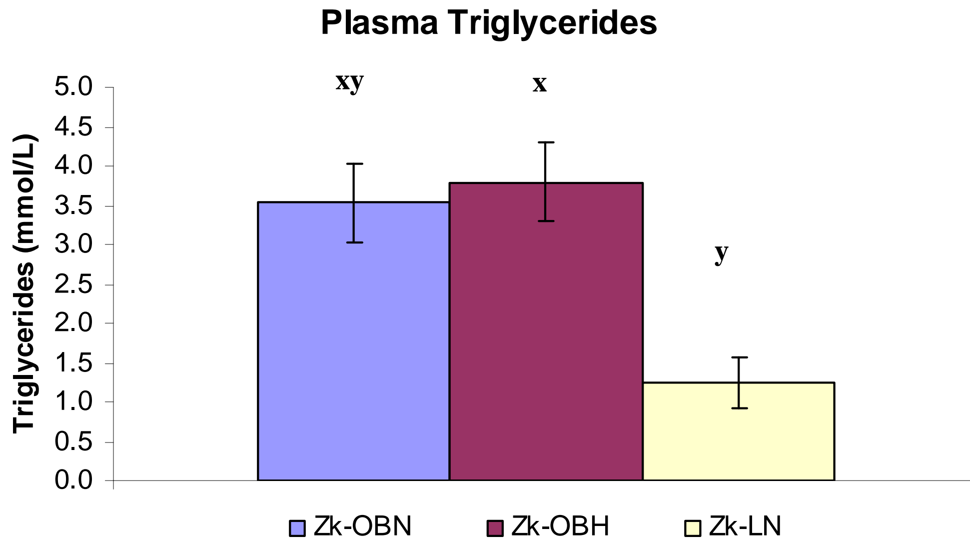

Figure 14. Plasma triglyceride levels after outlier removal in Zucker Obese and Lean rats after receiving B_6 treatment

Values are expressed as mean ± SE. An outlier was removed from Zk-OBH, therefore N=7. N=8 Zk-OBN and N=6 for Zk-LN. Bars without a common superscript are significantly different ($P<0.05$, ANOVA analysis with LSD post hoc tests). Abbreviations: Zk-OBN, Zucker Obese normal B_6 group; Zk-OBH, Zucker Obese high B_6 group; Zk-LN – AOM-injected Zucker Lean rats receiving normal B_6.

4.3.4 Liver Cholesterol Levels

To compare the concentration of cholesterol in hepatic tissue of Zucker Obese and Lean rats, an equal amount of protein totaling 50 μg was loaded for all samples. Seven samples of liver tissue from each Obese treatment group were selected for assay while six samples were selected for the Zucker Lean group. Significant differences in liver cholesterol concentration were not observed between Zucker Obese rats receiving normal and high levels of pyridoxine (Figure 14). Moreover, significant differences in liver cholesterol were not observed between the Zucker Lean group and either of the Obese treatment groups.

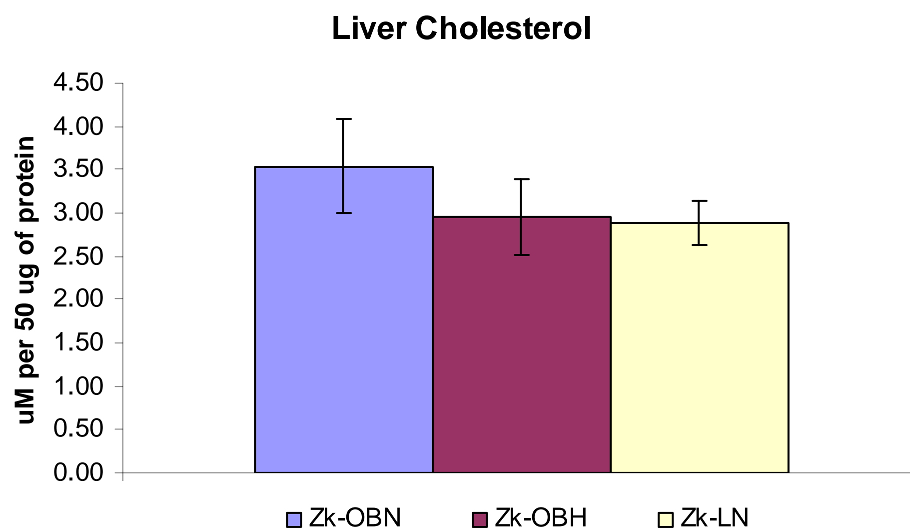

Figure 15. Liver cholesterol levels in Zucker Obese and Lean rats after receiving B_6 treatment.

Liver cholesterol levels were assayed using the Amplex Red kit by fluorometric detection. For each sample, a volume equal to 50 µg of protein was used to detect cholesterol level. All values are mean ± SE. N=7 for Zucker Obese dietary treatment groups and N=6 for the Zucker Lean group. Abbreviations: Zk-OBN, Zucker Obese rats receiving normal levels of vitamin B_6; Zk-OBH, Zucker Obese rats receiving high vitamin B_6; Zk-LN, AOM-injected Zucker Lean group receiving normal vitamin B_6.

4.3.5 Enumeration of Aberrant Crypt Foci (ACF)

Six weeks following the second AOM injection and after ten weeks of dietary treatment, the colons of Zucker Obese and Lean rats were fixed and examined for preneoplastic changes. The results of the quantification of ACF are summarized in Table 21. Zucker Obese rats receiving the normal B_6 diet had a significantly higher amount of total ACF compared to the high B_6 treatment group and Zucker lean animals ($P \leq 0.05$). Furthermore, the Obese group receiving normal B_6 had significantly higher enumeration of primal (1-3 crypts) and intermediate ACF (4-6 crypts) compared to the Zucker Obese high B_6 and Zucker Lean treatment groups ($P \leq 0.05$). The number of total crypts, primal and intermediate ACF were similar between the high B_6 Zucker Obese group and Zucker Lean rats. Differences were not observed in the enumeration of advanced ACF (7+ crypts) between any of the animal groups.

Table 21. Enumeration of ACF growth characteristics in AOM-injected female Zucker Obese and Lean rats after receiving vitamin B_6 treatment[a]

Group	Total ACF	Primary ACF (1-3 Crypts)	Intermediate ACF (4-6 Crypts)	Advanced ACF (7+ Crypts)
Zk-OBN	209.6 ± 19.5^{x}	194.9 ± 17.7^{x}	14.4 ± 2.5^{x}	0.4 ± 0.3^{x}
Zk-OBH	151.3 ± 18.9^{y}	141.6 ± 17.7^{y}	9.4 ± 1.4^{y}	0.3 ± 0.2^{x}
Zk-LN	135.0 ± 16.9^{y}	128.6 ± 16.4^{y}	6.3 ± 1.4^{y}	0.2 ± 0.2^{x}

[a]Values are expressed as mean ± SE. N=8 per Zucker Obese group and N=7 for Zk-LN. Values in the same column without a common superscript are significantly different ($P < 0.05$, ANOVA analysis with LSD post hoc tests). Abbreviations: ACF, aberrant crypt foci; Zk-OBN, Zucker Obese normal B_6 group; Zk-OBH, Zucker Obese high B_6 group; Zk-LN, AOM-injected Zucker Lean group receiving normal B_6.

4.3.6 Plasma PLP, Homocysteine and Cysteine Measurement

Plasma of Zucker Obese rats was analyzed for PLP levels using a ^{3}H-tyrosine radioenzymatic assay kit. Challenges were faced bleeding the animals during termination and a limited amount of blood was retained from each animal. As a result, plasma from Zucker Lean rats was unavailable for PLP analysis. Significant differences were not observed in PLP levels between Zucker Obese normal and high B_6 treatment groups (Figure 15).

Plasma samples of the Zucker Obese treatment groups and the Zucker Lean group were analyzed for homocysteine and cysteine by reverse phase HPLC as previously described (Table 22). After sending an adequate amount of plasma to Guelph Animal Laboratories for CBC and biochemical analysis, only four samples remained per Zucker Obese treatment group and three for the Zucker Lean group. A comparison of homocysteine concentration in Zucker Obese rats receiving normal B_6 and high B_6 did not indicate significant differences. However, the Zucker Lean animal group had a significantly higher concentration of homocysteine compared to both Zucker Obese groups ($P \leq 0.05$). Significant differences in the concentration of plasma cysteine were not observed between any of the Zucker Obese treatment groups and the Zucker Lean animal group.

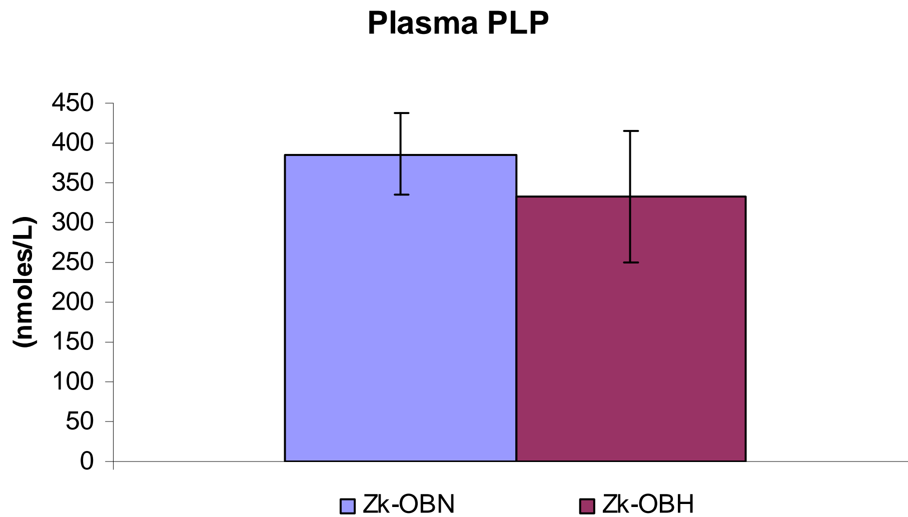

Figure 16. Plasma PLP in AOM-injected female Zucker Obese rats after receiving vitamin B_6 treatment

Plasma PLP was measured using a commercially available radioenzymatic assay kit. All values are mean ± SE, N=4 per dietary group. Abbreviations: PLP, pyridoxal 5'-phosphate; Zk-OBN, Zucker Obese rats receiving normal vitamin B_6; Zk-OBH, Zucker Obese rats receiving high Vitamin B_6.

Table 22. Plasma cysteine and homocysteine concentrations in female AOM-injected Zucker Obese and Lean rats after vitamin B_6 treatment[a]

Parameter	Zk-OBN	Zk-OBH	Zk-LN
Homocysteine (µM)	2.87 ± 0.06^{x}	3.09 ± 0.51^{x}	6.21 ± 0.83^{y}
Cysteine (µM)	184.9 ± 6.0^{x}	167.2 ± 13.3^{x}	193.9 ± 13.3^{x}

[a]Plasma homocysteine and cysteine levels in Zucker Obese and Lean were assayed by reverse phase HPLC analysis. All values are mean ± SE. As a result of challenges experienced in bleeding the animals, a limited amount of plasma was available for analysis. Therefore, for cysteine and homocysteine analyses N=4 per Zucker Obese dietary group and N=3 for the Zucker Lean group. Values within a row without a common superscript (x, y) differ significantly ($P<0.05$, ANOVA analysis with LSD post hoc tests). Abbreviations: Zk-OBN, Zucker Obese rats receiving normal levels of vitamin B_6; Zk-OBH, Zucker Obese rats receiving high Vitamin B_6; Zk-LN, AOM injected Zucker Lean group receiving normal vitamin B_6.

4.3.7 Antioxidant Status

The antioxidant status of Zucker Obese and Lean rats treated with normal and high levels of vitamin B_6 was assessed through a measurement of hepatic levels of GSH, NO_2^-, antioxidant enzymes SOD, GPx and CAT. To determine the activities of antioxidant enzymes, hepatic tissue samples were homogenized in their respective buffers and equal sample volumes were analyzed.

To measure the level of GSH in hepatic tissue, an equal volume of whole liver extract was assayed for each sample. The results of the GSH assay are demonstrated in Figure 16. Seven liver samples for each treatment group were used for the assay. Zucker Obese rats receiving a diet high in vitamin B_6 had significantly higher levels of GSH compared to counterparts receiving normal B_6 ($P<0.05$). Interestingly, the level of GSH in Zucker Lean animals was similar to the Zucker group treated with high B_6.

NO_2^- levels were measured as a marker for nitric oxide. Plasma of Zucker Obese rats was analyzed for nitrite levels (Figure 17A). Due to difficulties experienced in bleeding the animals at termination, only four plasma samples from the normal B_6 group (Zk-OBN) and give samples from the high B_6 group (Zk-OBH) were available for analysis. As a result of sending plasma samples for CBC, biochemical, homocysteine and cysteine analysis, plasma samples representative of the Zucker Lean group were unavailable for NO_2^- analysis. While not significant, Zucker Obese rats receiving normal B_6 had higher levels of plasma nitrite compared to the high B_6 group. Whole extracts of hepatic tissue from both Zucker Obese treatment groups and the Zucker Lean group were assayed for nitrite level (Figure 17B). Six samples were selected from each treatment group. A similar trend observed for plasma NO_2^- results between the normal and high B_6 groups was observed in hepatic tissue; the ZK-OBN group had a significantly higher level of hepatic nitrite ($P\leq0.05$). Furthermore, Zucker Lean rats had significantly higher levels of hepatic NO_2^- than both Zucker Obese treatment groups ($P\leq0.05$).

For the SOD assay, six samples of hepatic tissue were used from each Zucker Obese treatment group. All eight livers from the Zucker Lean group were used. Significant

differences were not observed in the activities of SOD between Zucker Obese and Lean treatment groups (Figure 18). For GPx and CAT activity assays, all eight livers were used from both Zucker Obese treatment groups. Livers of AOM-injected Zucker Lean rats were unavailable for GPx and CAT assay. Instead livers from non-injected Zucker Lean rats (lean control group) were used for comparison to activity in Zk-OBN and Zk-OBH rats. The results of the activities of GPx and CAT are summarized in Table 23. The activity of GPx in Zk-OBN was significantly higher than Zk-OBH and Zk-LC ($P \leq 0.05$). Significant differences in CAT activity were not observed between both Zucker Obese treatment groups and the Zucker Lean control rats.

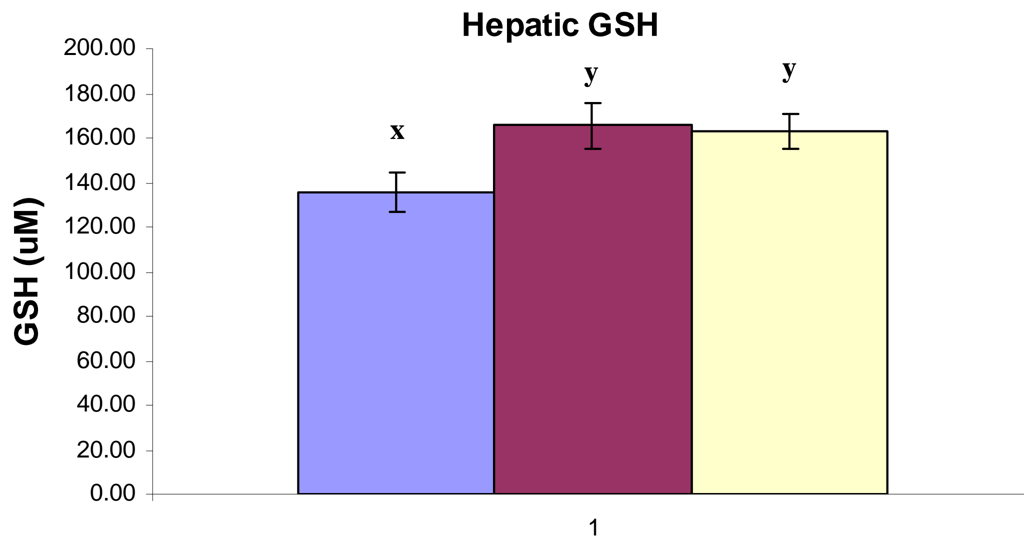

Figure 17. Colorimetric determination of glutathione (GSH) in whole extracts of liver from female AOM-injected Zucker Obese and Lean rats after receiving vitamin B_6 treatment.

GSH in liver tissue was assayed for Zucker Obese normal and high vitamin B_6 treatment groups and Zucker Lean rats. All values are mean ± SE, N=7 per dietary group. Bars without a common letter (x, y, z) differ significantly, $P<0.05$, ANOVA analyses with LSD post hoc tests. Abbreviations: GSH, glutathione; Zk-OBN, Zucker Obese rats receiving normal levels of vitamin B_6; Zk-OBH, Zucker Obese rats receiving high vitamin B_6; Zk-LN, AOM-injected Zucker Lean group receiving normal vitamin B_6.

Figure 18. Colorimetric determination of nitrite in plasma and whole extracts of liver from female AOM-injected Zucker Obese and Lean rats after receiving vitamin B_6 treatment

All values are mean ± SE. (A) Represents results of plasma nitrite measured in Zucker Obese treatment groups, Zk-OBN and Zk-OBH. Due to challenges with bleeding the animals at termination, N=4 for Zk-OBN and N=5 for Zk-OBH for plasma NO_2^- measurement. Plasma from Zucker Lean rats was not available for analysis. (B) Represents hepatic nitrite levels in Zucker Obese and Zucker Lean treatment groups. N=6 for liver NO_2^- measurement. Bars without a common letter (x, y, z) differ significantly, $p \leq 0.05$ as determined by ANOVA analyses in conjunction with LSD post-hoc tests. Abbreviations are as follows: Zk-OBN, Zucker Obese rats receiving normal levels of vitamin B_6; Zk-OBH, Zucker Obese rats receiving high levels of B_6; Zk-LN, AOM-injected Zucker lean group receiving normal vitamin B_6; NO_2^-; nitrite.

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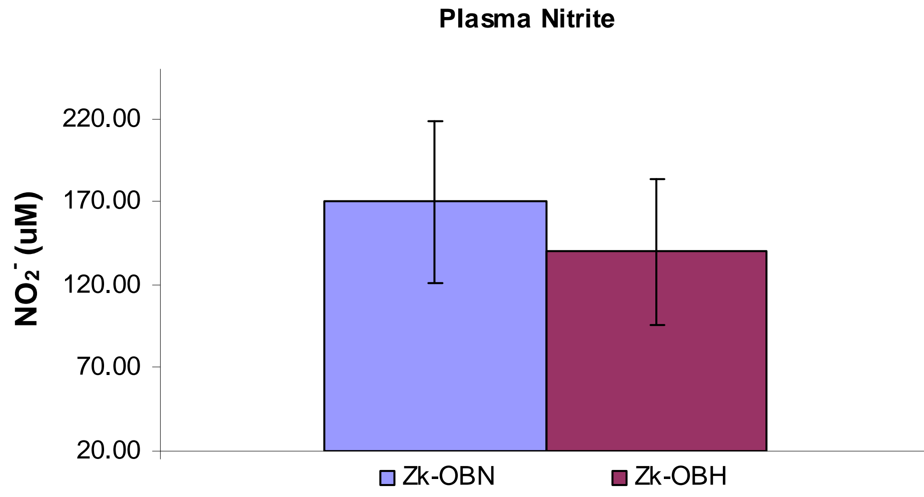

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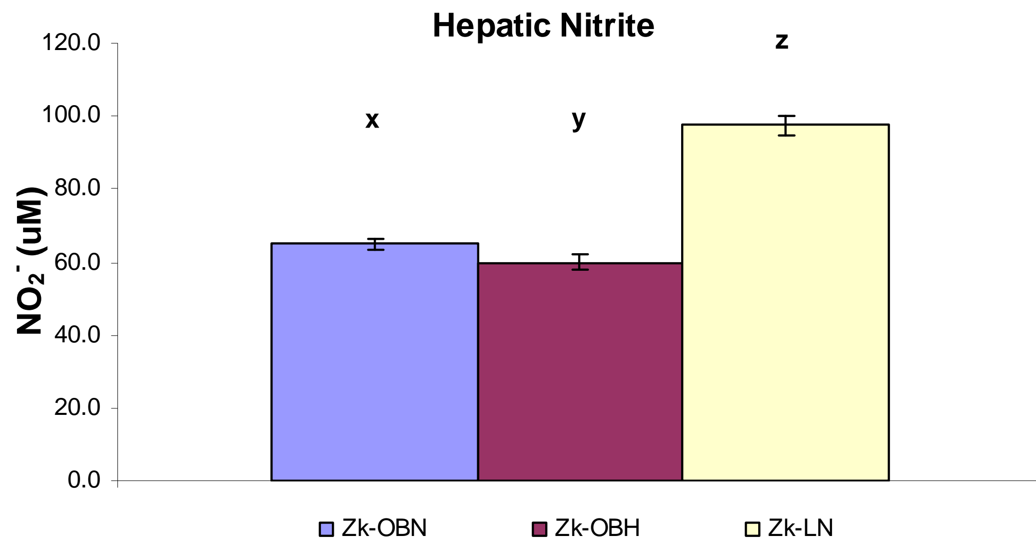

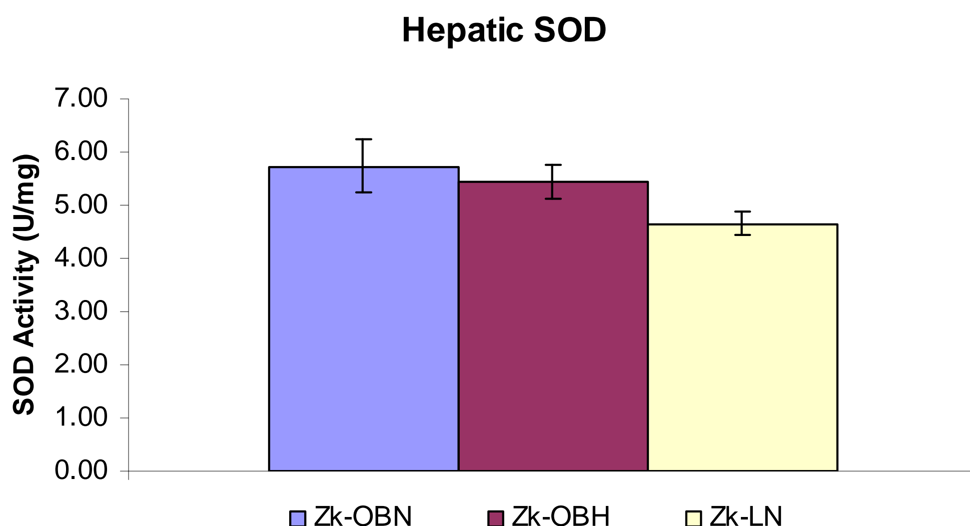

Figure 19. SOD activity in hepatic tissue of female AOM-injected Zucker Obese and Lean rats after receiving vitamin B_6 treatment.

Values are expressed as mean ± SE. N=6 per Zucker Obese dietary treatment group and N=8 for the Zucker Lean group. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Abbreviations: Zk-OBN, Zucker Obese normal B_6 group; Zk-OBH, Zucker Obese high B_6 group; Zk-LN, AOM-injected Zucker Lean group receiving normal vitamin B_6; SOD, superoxide dismutase.

Table 23. GPx and CAT activities in hepatic tissue[1,2,3,4,5] of female AOM-injected Zucker Obese rats and non-injected Zucker Lean rats after receiving vitamin B_6 treatment

Antioxidant Enzyme	Zk-OBN	Zk-OBH	Zk-LC
GPx Activity (U/mg)	227.1 ± 16.8^{x}	168.3 ± 12.6^{y}	176.67 ± 90.1^{xy}
CAT Activity (U/mg)	1037.65 ± 102.6^{x}	1087.9 ± 92.1^{x}	1425.7 ± 320.0^{x}

1. Values are expressed as mean ± SE. For GPx and CAT activities N=8 per Zucker Obese dietary groups and N=4 for the Zk-LC group.
2. Values in the same row not sharing a common superscript are significantly different ($P \leq 0.05$, ANOVA analyses with LSD post hoc tests).
3. One unit of GPx activity is expressed as the amount of enzyme that would catalyze the oxidation of 1.0 nmol NADPH to $NADP^+$ per minute at 25°C.
4. For catalase activity one unit is defined as the amount of enzyme that will cause formation of 1.0 nmol of formaldehyde per minute at 25°C.
5. Abbreviations: Zk-OBN, Zucker Obese normal vitamin B_6 group; Zk-OBH, Zucker Obese high vitamin B_6 group; Zk-LC, control group –non-injected Zucker Lean rats; GPx, glutathione peroxidase; CAT, catalase.

4.4 Discussion

Study 2 was undertaken to determine if treatment with supplementary pyridoxine would reduce obesity-related oxidative stress and thereby suppress colon carcinogenesis. To our knowledge, it was the first study to examine the effect of supplementary pyridoxine against colon carcinogenesis in an obese state. Zucker Obese rats were fed high B_6 two weeks prior to carcinogen injection to improve the state of the colon. It was hypothesized that this would reduce oxidative stress and inflammation during initiation and thereby suppress carcinogenesis. Using ACF as a biomarker, results from the study did show that pyridoxine was suppressing the promotion of early colon carcinogenesis. Zucker Obese rats receiving high B_6 had lower numbers of total, primal and intermediate ACF compared to their normal B_6 counterparts. Moreover, a high vitamin B_6 diet modulated a number of parameters in hepatic tissue and plasma, suggesting a favorable effect.

The liver accounts for 10% of the total body pool of PLP and is the primary organ responsible for metabolism of B_6 vitamers (Merrill et. al., 1984). High concentrations of antioxidants and antioxidant enzymes occur within the liver. In addition to plasma, liver tissue was used to examine the antioxidant capacity of pyridoxine in an obese animal model. The redox state of liver tissue was assessed by evaluating levels of nitric oxide, GSH and measuring activities of antioxidant enzymes, SOD, CAT and GPx. There was no effect by supplementary vitamin B_6 on SOD and CAT in Zucker animals. However, hepatic GSH levels increased while GPx activity decreased in Zucker Obese rats receiving supplementary vitamin B_6. The concentration of GSH has a major effect on its antioxidant function; GSH concentrations have been observed to decrease when oxidative stress increases. Furthermore, a reduction in GPx activity in the high B_6 group may suggest lower oxidative stress. Together, these results suggest that high vitamin B_6 is exerting a protective effect.

In the present study, nitrite served as a marker for NO. NO is a highly reactive free radical. There is conflicting evidence on the concentrations of NO and its role in carcinogenesis (Pervin et. al., 2007). Studies have shown that NO can both promote and inhibit tumor progression and metastasis. In general, NO and its derivatives induce oxidative and nitrosative stress that causes DNA damage and inhibits activity of DNA repair enzymes

(Seifried et. al., 2007). Since the level of nitrite in the colon could not be determined due to previous staining with methylene blue, we could not assume its role in the promotion of early carcinogenesis. However, the concentration of nitrite in plasma and liver tissue was measured. Zucker Obese rats receiving high B_6 had reduced production of hepatic nitrite compared to the normal B_6 group. While not significant, plasma analyzed for NO production also indicated lower levels in the high B_6 group compared to the normal B_6 group. Comparing the two obese treatment groups alone, it would appear that supplementary B_6 is having a protective effect by decreasing NO production and subsequently decreasing the opportunity for nitrosative-dependent cellular damage in the liver. In combination with lower liver weights, one can suggest that high B_6 was ameliorating the state of the organ. The level of hepatic nitrite in Zucker Lean rats was higher than quantities measured in both Zucker Obese groups. Similar results were found in Zucker Lean rats not injected with azoxymethane. It could be possible that the large differences in NO synthesis between the obese and lean groups could be attributed to differences in the phenotypes. Zucker Obese rats are susceptible to different physiological stresses that are due to hyperphagia, including increased fat accumulation, high glucose, hyperinsulinemia and inflammation. However, based on the overall findings in this study and the complexity of the NO signaling network and its effects, further experimental analysis on the tissues could be performed to better speculate the role of NO at this stage of disease progression and in each phenotype. This might include measuring protein expression of NOS isoforms (iNOS and eNOS) in hepatic and colon tissue.

Vitamin B_6 is a coenzyme of the transulfuration pathway that metabolizes homocysteine, generating cysteine. The biosynthesis of GSH is limited by the availability of cysteine. By treating Zucker Obese rats with high B_6 it was expected that there would be an increase in circulating vitamin B_6 in plasma and localized in tissues. Increased physiological concentrations of vitamin B_6 should translate to its greater use as a co-factor for PLP-dependent enzymes that participate in the transulfuration pathway. In theory, this would decrease homocysteine and increase cysteine. The concentration of plasma PLP in the Zucker Obese high B_6 group did not vary significantly from the normal B_6 group. High B_6 also had no affect on the concentrations of plasma homocysteine and cysteine. Previous studies in rats

suggest that plasma PLP levels do not provide a representative indication of vitamin B_6 status (Schaeffer et. al., 1989). Therefore, we can not conclude that systemically, high B_6 intake is not having an effect on vitamin B_6 status. However, the findings do indicate that high vitamin B_6 is not exerting an effect on carbon-1 metabolism in plasma. As previously noted GSH levels in hepatic tissue of the high B_6 group increased. Provided that the liver is the primary organ for vitamin B_6 metabolism, we could assume that at a supraphysiological dose more B_6 was localized at this site and available as a coenzyme for GSH synthesis.

White blood cells are increased during inflammation. It appears that supplementary vitamin B_6 was exerting an effect by lowering concentrations of monocytes and lymphocytes. Given the intimate relationship between inflammation and cancer, further research would be necessary to determine how vitamin B_6 is exerting an effect and the implications. This would include analyzing changes in markers for inflammation between the two B_6 treatment groups. Supplementary vitamin B_6 treatment also significantly reduced platelet count. Activation and aggregation of platelets are key events in the pathophysiology of thrombosis, atherosclerosis, inflammation and cancer (Ginsberg et. al., 1988, Gasic et. al., 1968., Jurasz et. al., 2004). Evidence suggests that decreased platelet counts reduce metastasis (Gasic et. al., 1968). Cancer cells have been shown to aggregate platelets conferring a selective advantage for survival and spread. Tumour-platelet aggregates help tumour cells evade the immune system, protect them from the sheer forces of flowing blood, embolize the microvasculature at new sites and facilitate adhesion to vascular endothelium (Jurasz et. al., 2004). In addition, platelets can secrete growth factors that can be used for tumour cell proliferation (Honn and Tang, 1992). Vitamin B_6 has been shown to inhibit platelet aggregation and suggested as a compound that should be included in the design of novel antiplatelet agents (Chang et. al., 2002 and Zhang et. al., 2004). Given a reduction of platelet count by supplementary vitamin B_6, previous evidence of the vitamer's ability to reduce aggregation and deleterious effects of the platelet-tumour relationship, one can suggest that this is a potential mode for supplementary pyridoxine's ability to suppress carcinogenesis.

In addition to evaluating the effect of supplementary vitamin B_6 on colon cancer promotion and state of oxidative stress, changes in the liver were also noted. The liver weights of the Zucker Obese high B_6 group were lower than counterparts receiving normal B_6. During

termination, visual differences in the pigmentation of the livers between the groups were also noted. In general, livers of the Zucker Lean group and Sprague-Dawley alike present deep red livers at termination, reflecting erythrocyte saturation. In contrast, the gross morphology of the Zucker Obese liver is observed to be marble-like with yellow coloration; a classic indicator of a steatotic liver (Raju and Bird, 2006). At termination, the livers of the high B_6 group appeared darker in pigmentation; a more intense pink/red colour compared to the normal B_6 group. Changes in liver weight and pigmentation suggest that supplementary pyridoxine might be modulating non-alcoholic hepatic steatosis in Zucker Obese rats. Non-alcoholic steatosis is described as the accumulation of triglycerides within the cytoplasm of hepatocytes which often results in impairment of hepatic microcirculation (Ijaz, 2005). *In vivo* studies of animals with steatotic livers have indicated that fatty infiltration reduced total hepatic blood flow and hepatic parenchymal microcirculation (Seifalian et. al., 1999 and Ijaz et. al., 2005). Studies with Zucker Obese rats have reported an undetected intersinusoidal erythrocyte flow compared to control rats (Sato et. al., 1999).While the livers of the high B_6 group did not appear as dark as a Zucker Lean (Fa/Fa) liver, a healthier pigmentation was observed. Therefore, one can infer that vitamin B_6 is increasing vascular circulation and intersinusoidal blood flow in hepatic tissue. The mechanism by which B_6 could increase hepatic circulation is unclear. However, pyridoxine is a co-factor in a number of important metabolic pathways including lipid metabolism, and alteration by greater availability of the B_6 coenzyme would account for the darker pigmentation. Future investigation including histological analysis of hepatic tissue by staining with Oil Red O (ORO) would confirm if there were hepatic changes in lipid accumulation.

In addition to lowering liver weight, high B_6 reduced plasma cholesterol, another hallmark of obesity. High levels of homocysteine, C-reactive protein, triglycerides and cholesterol are all strongly associated with cardiovascular disease (LaRosa, 1986 and Lin et. al., 2006). While supplementary pyridoxine had no effect on plasma homocysteine and triglycerides, this finding suggests that high vitamin B_6 may be ameliorating the risk of developing cardiovascular disease by lowering plasma cholesterol.

In conclusion, at a supraphysiological dose, vitamin B_6 exerted favorable effects in the Zucker Obese animal model. The findings of this study support the contention that an abnormal metabolic state which includes obesity and hyperglycemia may increase the need for vitamin B_6 and that at a high dose it could function as an antioxidant. It is believed that continued consumption of supplementary vitamin B_6 would decrease tumour incidence by not only attenuating oxidative stress, but by also maintaining lower platelet counts and suppressing the cancer-promoting effects of tumour-platelet aggregation.

Chapter 5
General Discussion and Conclusion

The main objective of this study was to explore if vitamin B_6 plays any role in colon carcinogenesis. Epidemiological and experimental studies have supported the notion that low B_6 status may be causally related to an increased risk for developing colon cancer. A review of the literature regarding the physiological function of vitamin B_6, in addition to its classical role in amino acid metabolism, suggests that it plays a role in lipid metabolism and also may act as an antioxidant (Kannan and Jain, 2004). The recommended dose of vitamin B_6 is based on its classical function involving amino acid metabolism. Vitamin B_6 status is generally assessed by direct measurement of the level of plasma PLP, total vitamin B_6 and homocysteine. Whether vitamin B_6 functions as antioxidant *in vivo* remained to be explored. In the present research, two studies were conducted. The first study was conducted in male Sprague Dawley rats, using three levels of vitamin B_6 (low, normal and high) and two different levels of protein (normal and high). We postulated that if indeed vitamin B_6 functions as an antioxidant then a high protein and low B_6 diet would increase the oxidative stress. Moreover, it was further hypothesized that a low B_6 intake would enhance the number and growth of early preneoplastic lesions, ACF, while high B_6 would suppress their growth. To determine the antioxidant status of the animals a number of measurements were made at the plasma and hepatic level.

It was interesting to note that in the high protein and low B_6 group the liver weight was moderately higher than the other groups and, unexpectedly, the number and growth of ACF were lowest in this group. The results of ACF enumeration did not support the conjecture that a low B_6 intake would augment the risk of developing colon cancer. As a matter of fact, in the other groups, a similar number of ACF were enumerated regardless of the level of B_6 or protein treatment. When the dietary variables were scrutinized, it became apparent that the level of protein was an important variable. The high protein groups generally exhibited lower levels of oxidative stress markers and plasma levels of cholesterol and triglycerides. In the high protein groups, the additional protein was added at the expense of an equal amount of carbohydrate. One can suggest that, in part, the differences noted between the normal and

high protein groups were due to the differences in the level of carbohydrate. Therefore, the findings did not support the hypothesis that a low B_6 diet would enhance or a high B_6 intake would reduce the risk of developing colon cancer. In contrast, our findings suggest that preneoplastic lesions need B_6 for optimal growth. The low B_6 diet in the normal protein group did not show any sign of B_6 deficiency suggesting that, in order to induce deficiency, a longer duration may be required or the low B_6 used in our study may be inducing only a border line compromised status.

In the second study, a completely different animal model was used. We used Zucker Obese rats known to be a good model of human obesity. Zucker Obese rats exhibit a number of physiological abnormalities including elevated levels of pro-inflammatory cytokines and increased oxidative stress. These animals are susceptible to liver steatosis, diabetes and colon cancer. It is hypothesized that the reason these animals are highly sensitive to developing colon cancer is because they are in a chronic state of inflammation, a condition known to enhance the risk of colon cancer. We wanted to determine if the sensitivity of these animals to colon carcinogenesis could be modified if vitamin B_6 intake was increased. The rational for this study was that supplementary vitamin B_6 might alleviate the oxidative stress response *in vivo*. It was interesting to note that not only did a high B_6 diet reduce the number of ACF, but a decrease in a number of physiological parameters were noted compared to the normal vitamin B_6 group. Zucker Obese rats exhibit liver steatosis and hepatomegaly. In this study, liver weights in the high B_6 group were significantly lower than their normal B_6 counterparts. The number of monocytes, platelets and lymphocytes were also lower in the high B_6 group. Hepatic GSH level increased in the high B_6 group with a concomitant decrease in glutathione peroxidase activity compared to the normal B_6 group. These findings supported the notion that a pharmacological dosage of B_6 was beneficial to the obese rats.

The study conducted in the Zucker Obese rats has several limitations. We were unable to collect sufficient blood samples to conduct a complete analysis of oxidative stress markers at the plasma level. As a pilot study, the sample size was kept small and, as a result, all of the colons were stained for crypt enumeration. In the future, the sample size could be increased to accommodate the need to investigate levels of oxidative stress and inflammatory markers in colon tissue.

The two studies differ a great deal from each other; therefore, a direct comparison between the findings of the two studies can not be made. Nevertheless, both studies raise important questions. The findings of the second study are interesting and suggest that in future study, the role of vitamin B_6 in pre- and post-initiation stages of carcinogenesis must be conducted. By increasing the duration of the study period from six to 24 weeks, the effect of supplementary vitamin B_6 on tumour incidence could be determined. It is also important to determine and establish if vitamin B_6 is indeed reducing inflammation or if it has some other role when ingested at a dose normally not achievable by diet. The effect of high B_6 on plasma cholesterol and other blood constituents were not expected and warrant further investigation.

This research has provided evidence to consider vitamin B_6 as a nutraceutical with a potential to be beneficial in a metabolic compromised state of obesity.

Appendix A

ABBREVIATIONS

ACF	aberrant crypt foci
ALT	alanine aminotransferase
AOM	azoxymethane
Apc	adenomatous polyposis coli
AST	aspartate aminotransferase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CAT	catalase
CBC	complete blood count
CBS	cystathionine β-synthase
Cd	cadmium
CGS	cystathionine γ-lyase
CK	creatine kinase
CE	cholesterol esterase
CO	cholesterol oxidase
Cu	copper
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
FAP	familial polyposis
GLDH	glutamate dehydrogenase
GK	glycerol kinase
GOD	glucose oxidase
GPx	glutathione peroxidase

GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulphide
H_2O_2	hydrogen peroxide
Hb	hemoglobin
HCT	hematocrit
HDL	high density lipoprotein
HK	hexokinase
HPLC	high-performance liquid chromatography
HPNC	hereditary non polyposis colorectal cancer
HRP	horseradish peroxidase
iNOS	inducible nitric oxide synthase
LDL	low density lipoprotein
LDH	Lactate dehydrogenase
LF	low fat
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MDH	malate dehydrogenase
MethylTHF	N-5-methyltetrahydrofolate
MethyleneTHF	N-5-methylenetetrahydrofolate
MPV	mean platelet volume
NAC	N-acetylcysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
PL	pyridoxal
O^{2-}	superoxide anion

PLP	pyridoxal 5’-phosphate
PMP	pyridoxamine 5’-phosphate
PN	pyridoxine
PN-HCl	pyridoxine hydrochloride
RBC	red blood cell count
RDW	red cell distribution width
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-methionine
SOD	superoxide dismutase
SD	Sprague-Dawley rats
THF	tetrahydrofolate
VLDL	very low density lipoprotein
WBC	white blood cell count
Zk-LC	Zucker lean rats, control group
Zk-LN	Zucker lean rats, AOM-injected
Zk-Ob	Zucker Obese rats
Zk-OBH	Zucker Obese rats, AOM injected, receiving 35 mg/kg of pyridoxine-hydrochloride
Zk-OBN	Zucker Obese rats, AOM injected, receiving 7 mg/kg of pyridoxine-hydrochloride

Appendix B

Diet Compositions

Table B 1: Composition of AIN-93 G experimental diets in g/Kg for Study 1 - Modified with respect to casein and vitamin B_6 for Sprague-Dawley rats

	Normal Protein (20%)			High Protein (40%)		
Ingredients	**Low B_6**	**Nor B_6**	**High B_6**	**Low B_6**	**Nor B_6**	**High B_6**
Casein	**200**	**200**	**200**	**400**	**400**	**400**
L-Cystine	3.0	3.0	3.0	3.0	3.0	3.0
Corn Starch	417.336	417.336	417.336	214.536	214.536	214.536
Maltodextrin	132.0	132.0	132.0	132.0	132.0	132.0
Sucrose	109.74	109.74	109.74	109.74	109.74	109.74
Corn Oil	50.0	50.0	50	50.0	50.0	50.0
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0
Mineral Mix AIN-93-G	35.0	35.0	35.0	35.0	35.0	35.0
Calcium Carbonate	-	-	-	5.8	5.8	5.8
Niacin	0.03	0.03	0.03	0.03	0.03	0.03
Calcium Pantothenate	0.016	0.016	0.016	0.016	0.016	0.016
Pyridoxine-HCl	**0.0035**	**0.007**	**0.014**	**0.0035**	**0.007**	**0.014**
Thiamin HCl	0.006	0.006	0.006	0.006	0.006	0.006
Riboflavin	0.006	0.006	0.006	0.006	0.006	0.006
Folic Acid	0.002	0.002	0.002	0.002	0.002	0.002
Biotin	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002
Vitamin B_{12} (0.1% in mannitol)	0.025	0.025	0.025	0.025	0.025	0.025
Vitamin A Palmitate	0.008	0.008	0.008	0.008	0.008	0.008
Vitamin D_3, cholecalciferol	0.002	0.002	0.002	0.002	0.002	0.002
DL-Alpha Tocopheryl Acetate	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin K, phylloquinone	0.0008	0.0008	0.0008	0.0008	0.0008	0.0008
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
TBHQ (antioxidant)	0.014	0.014	0.014	0.014	0.014	0.014

Table B 2: Composition of AIN-93 G experimental diets in g/Kg for Study 2 - Modified with respect to vitamin B_6 for Zucker Obese and Lean rats[a]

Ingredients	Zk-OBN	Zk-OBH	Zk-LN	Zk-LC
Casein	400.0	400.0	400.0	400.0
L-Cysteine	3.0	3.0	3.0	3.0
Corn Starch	214.536	214.536	214.536	214.536
Maltodextrin	132.0	132.0	132.0	132.0
Sucrose	109.74	109.74	109.74	109.74
Corn Oil	50.0	50.0	50.0	50.0
Cellulose	50.0	50.0	50.0	50.0
Mineral Mix AIN-93-G	35.0	35.0	35.0	35.0
Calcium Carbonate	5.8	5.8	5.8	5.8
Niacin	0.03	0.03	0.03	0.03
Calcium Pantothenate	0.016	0.016	0.016	0.016
Pyridoxine-HCl	**0.007**	**0.035**	**0.007**	**0.007**
Thiamin HCl	0.006	0.006	0.006	0.006
Riboflavin	0.006	0.006	0.006	0.006
Folic Acid	0.002	0.002	0.002	0.002
Biotin	0.0002	0.0002	0.0002	0.0002
Vitamin B_{12} (0.1% in mannitol)	0.025	0.025	0.025	0.025
Vitamin A Palmitate	0.008	0.008	0.008	0.008
Vitamin D_3, cholecalciferol	0.002	0.002	0.002	0.002
DL-Alpha Tocopheryl Acetate	0.15	0.15	0.15	0.15
Vitamin K, phylloquinone	0.0008	0.0008	0.0008	0.0008
Choline Bitartrate	2.5	2.5	2.5	2.5
TBHQ (antioxidant)	0.014	0.014	0.014	0.014

[a]Abbreviations: Zk-OBN, Zucker Obese normal B_6 group; Zk-OBH, Zucker Obese high B_6 group; Zk-LN, AOM-injected Zucker Lean group receiving normal B_6; Zk-LC, control Zucker Lean group (non-injected) receiving normal B_6.

Appendix C

Tables and Figures

Table C1. Weekly mean body weights of AOM-injected male Sprague-Dawley rats during eight weeks of intervention with diets varying in protein and vitamin B_6[a]

	High Protein			Normal Protein		
	Low B_6 (g)	**Normal B_6 (g)**	**High B_6 (g)**	**Low B_6(g)**	**Normal B_6 (g)**	**High B_6 (g)**
Day 1	342.6±4.6^{x}	319.8±8.5^{y}	333.5±6.8^{xy}	321.4±3.5^{y}	337.0±10.8^{xy}	334.8±8.2^{xy}
Week 1	391.4±5.3^{x}	368.6±10.8^{x}	377.5±7.4^{x}	373.0±8.1^{x}	390.5±12.4^{x}	389.4±6.6^{x}
Week 2	423.9±5.4^{x}	396.8±11.0^{x}	401.5±9.3^{x}	401.1±9.5^{x}	422.6±14.2^{x}	421.1±7.6^{x}
Week 3	453.6±6.7^{x}	419.0±12.2^{y}	424.5±11.5^{xy}	425.3±12.0^{xy}	450.4±15.6^{xy}	451.4±8.9^{xy}
Week 4	479.4±8.0^{x}	454.1±11.0^{x}	455.5±14.0^{x}	454.6±14.7^{x}	491.5±20.6^{x}	479.6±9.9^{x}
Week 5	503.3 ± 8.6^{xz}	466.8±11.9^{xy}	456.8±12.8^{y}	487.0±16.3^{yz}	504.6±16.5^{z}	504.1±10.5^{z}
Week 6	530.0±9.8^{x}	483.8±11.4^{y}	497.5±16.8^{xy}	512.4±18.5^{xy}	529.5±19.0^{x}	531.0±13.3^{x}
Week 7	532.8±19.7^{xyz}	500.1±12.0^{xz}	497.5±18.4^{z}	515.4±18.7^{xyz}	547.1±18.2^{xy}	549.0±13.2^{y}
Week 8	550.0±14.9^{x}	498.6±11.6^{y}	501.6±18.5^{y}	521.2±20.1^{xy}	549.0±19.1^{x}	551.5±13.4x

[a]All values are expressed as mean ± SE, N=8 per dietary group.

Table C 2. Amount of diet consumed weekly by AOM-injected male Sprague-Dawley rats during eight weeks of intervention with diets varying in protein and vitamin B_6

	High Protein			Normal Protein		
	Low B_6 (g)	Normal B_6 (g)	High B_6 (g)	Low B_6(g)	Normal B_6(g)	High B_6(g)
Week 1	1584	1464	1442	1591	1567	1565
Week 2	1052	931	921	939	979	1009
Week 3	1501	1289	1278	1357	1357	1393
Week 4	1518	1296	1329	1363	1339	1404
Week 5	1510	1289	1354	1333	1404	1406
Week 6	1514	1298	1195	1396	1429	1422
Week 7	1530	1280	1314	1377	1427	1453
Week 8	1585	1312	1406	1351	1475	1444

Table C 3. Complete blood count analyses in AOM-injected male Sprague-Dawley rats after eight weeks of intervention with diets varying in protein and vitamin B_6[a]

	High Protein			Normal Protein		
	Low B_6	Normal B_6	High B_6	Low B_6	Normal B_6	High B_6
WBC (10^9/L)	12.9 ± 1.2^{xy}	13.5 ± 1.2^{x}	10.3 ± 0.9^{y}	12.7 ± 1.6^{xy}	13.4 ± 1.1^{xy}	12.3 ± 0.6^{xy}
RBC (10^12/L)	9.0 ± 0.1^{x}	9.0 ± 0.1^{xy}	8.5 ± 0.3^{y}	8.8 ± 0.1^{xy}	8.8 ± 0.1^{xy}	9.0 ± 0.1^{xy}
Hb (g/L)	160.0 ± 1.4^{x}	158.0 ± 1.9^{x}	158.8 ± 3.9^{x}	156.4 ± 1.7^{x}	155.3 ± 2.3^{x}	159.3 ± 1.8^{x}
HCT (L/L)	0.48 ± 0.00^{x}	0.49 ± 0.00^{x}	0.48 ± 0.01^{x}	0.49 ± 0.01^{x}	0.48 ± 0.01^{x}	0.49 ± 0.01^{x}
MCV (fL)	53.8 ± 0.8^{x}	54.0 ± 0.4^{x}	53.6 ± 0.6^{x}	54.9 ± 0.6^{x}	54.3 ± 0.6^{x}	54.5 ± 0.5^{x}
MCH (pg)	53.8 ± 0.8	54.0 ± 0.4	53.6 ± 0.6	54.9 ±0.6	54.3 ± 0.6	54.5 ±0.5
RDW (%)	12.7 ± 0.1^{xy}	12.4 ± 0.2^{x}	12.4 ± 0.2^{x}	12.7 ± 0.2^{xy}	13.1 ± 0.3^{y}	12.5 ± 0.1^{x}
MCHC (g/L)	329.9 ± 1.9^{x}	326.0 ± 2.4^{xy}	330.3 ± 1.9^{x}	323.4 ± 0.9^{y}	327.3 ± 2.3^{xy}	325.1 ± 1.8^{xy}
Platelet (10^9/L)	884.0 ± 65.0^{xy}	883.3 ± 25.6^{xy}	866.5 ± 38.9^{x}	935.3 ± 38.0^{xy}	1000.3 ± 41.4^{y}	953.1 ± 48.6^{xy}
MPV (fL)	6.3±0.1	6.1±0.1	6.3±0.1	6.4±0.1	6.6±0.1	6.6±0.1
Seg Neutrophil (10^9/L)	1.94±0.22	1.71±0.19	1.87±0.25	1.85±0.24	1.82±0.15	1.69±0.17
Lymphocyte Count (10^9/L)	10.3 ± 0.9^{x}	11.2 ± 1.1^{x}	7.3 ± 1.0^{y}	10.1 ± 1.3^{xy}	10.9±1.0x	9.8 ± 0.6^{y}
Monocyte Count (10^9/L)	0.29 ± 0.04^{xy}	0.21 ± 0.02^{xy}	0.24 ± 0.06^{x}	0.31 ± 0.05^{x}	0.28 ± 0.05^{xy}	0.40 ± 0.05^{y}
Eosinophil Count (10^9/L)	0.16 ± 0.02	0.18 ± 0.02	0.16 ± 0.02	0.19 ± 0.02	0.20 ± 0.03	0.22 ± 0.03
Basophil Count (10^9/L)	0.26 ± 0.02	0.25 ± 0.03	0.20 ± 0.04	0.23 ± 0.04	0.25 ± 0.04	0.22 ± 0.03

[a]All values are expressed as mean ± SE, N=8 per dietary group. Values in the same row not sharing a common superscript (x, y, z) are significantly different ($P \leq 0.05$, ANOVA analyses with LSD post hoc tests). Abbreviations: WBC, white blood count; RBC, red blood count; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume.

Table C4. Complete clinical status of plasma in AOM-injected male Sprague-Dawley rats after 8 weeks of intervention with diets varying in protein and vitamin B_6[a]

	High Protein			Normal Protein		
	Low B_6	Normal B_6	High B_6	Low B_6	Normal B_6	High B_6
Creatinine (umol/L)	9.1 ± 3.4^{x}	12.9 ± 2.8^{xy}	19.0 ± 3.4^{y}	19.6 ± 4.2^{xy}	11.5 ± 2.7^{y}	13.8 ± 3.2^{y}
Urea (mmol/L)	5.9 ± 0.2^{x}	5.6 ± 0.2^{x}	5.1 ± 0.3^{xy}	4.6 ± 0.3^{y}	5.1 ± 0.4^{xy}	5.1 ± 0.3^{xy}
HDL-C (mmol/L)	1.8 ± 0.1^{xy}	1.5 ± 0.1^{x}	1.6 ± 0.2^{x}	1.9 ± 0.2^{xy}	2.0 ± 0.2^{y}	2.0 ± 0.2^{y}
Cholesterol (mmol/L)	2.2 ± 0.2^{xy}	1.8 ± 0.1^{x}	1.9 ± 0.2^{x}	2.2 ± 0.2^{xy}	2.4 ± 0.2^{y}	2.4 ± 0.2^{y}
Glucose (mmol/L)	11.2 ± 1.0^{x}	10.6 ± 0.9^{x}	10.6 ± 0.9^{x}	10.4 ± 0.8^{x}	11.5 ± 1.0^{x}	12.0 ± 0.7^{x}
Triglycerides (mmol/L)	1.4 ± 0.4^{x}	0.8 ± 0.1^{y}	0.8 ± 0.1^{y}	1.4 ± 0.3^{x}	1.2 ± 0.1^{x}	1.7 ± 0.2^{x}
Total Bilirubin (umol/L)	3.8±0.4	3.3±0.3	3.4±0.4	3.6±0.3	5.6±1.2	3.9±0.3
Conjugated Bilirubin (umol/L)	1.0±0.0	1.0±0.0	1.1±0.1	1.0±0.0	1.5±0.5	1.0±0.2
AST (U/L)	103.3 ± 8.2^{xz}	99.6 ± 5.1^{xz}	109.0 ± 4.2^{x}	79.4 ± 5.3^{y}	92.8 ± 5.7^{z}	80.8 ± 4.0^{y}
ALT (U/L)	47.3 ± 5.3^{xy}	47.0 ± 3.3^{xy}	52.8 ± 4.8^{x}	37.5 ± 3.3^{y}	50.4 ± 6.0^{x}	40.3 ± 3.9^{xy}
CK (U/L)	157.4±9.5	150.5±10.3	181.8±43.9	143.5±10.5	162.0±14.3	126.5±9.0

[a]All values are expressed as mean ± SE, N=8 per dietary group. Values in the same row not sharing a common superscript (x, y, z) are significantly different ($P \leq 0.05$, ANOVA analyses with LSD post hoc tests). Abbreviations: HDL-C, high density lipoproteins; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CK, creatine kinase.

Table C5. Weekly weights of AOM-injected Zucker Obese and Lean rats and Control Zucker Lean rats after dietary treatment with vitamin B_6[a]

	Average Weekly Mass (g)			
	Zk-OBN	Zk-OBH	Zk-LN	Zk-LC
Week 1	358.8 ± 15.7^{x}	383.8 ± 19.8^{x}	194.8 ± 6.5^{y}	189.9 ± 3.2^{y}
Week 2	435.4 ± 7.1^{x}	448.5 ± 11.6^{x}	217.0 ± 6.7^{y}	208.5 ± 4.1^{y}
Week 3	470.6 ± 7.6^{x}	485.8 ± 13.3^{x}	228.4 ± 6.8^{y}	225.6 ± 6.2^{y}
Week 4	488.8 ± 7.6^{x}	499.8 ± 14.1^{x}	238.5 ± 7.2^{y}	236.0 ± 4.4^{y}
Week 5	492.6 ± 7.6^{x}	503.8 ± 15.9^{x}	241.8 ± 6.9^{y}	242.3 ± 5.0^{y}
Week 6	509.9 ± 7.4^{x}	526.5 ± 16.9^{x}	255.9 ± 7.0^{y}	249.7 ± 5.3^{y}
Week 7	526.5 ± 9.5^{x}	533.1 ± 13.2^{x}	265.1 ± 7.3^{y}	258.0 ± 5.5^{y}
Week 8	533.6 ± 10.5^{x}	540.4 ± 11.7^{x}	268.8 ± 7.4^{y}	264.0 ± 5.8^{y}
Week 9	544.8 ± 13.1^{x}	559.4 ± 10.9^{x}	276.3 ± 6.9^{y}	269.8 ± 6.3^{y}
Week 10	550.8 ± 13.5^{x}	557.2 ± 9.8^{x}	267.0 ± 8.4^{y}	NA

[a]All values are expressed as mean ± SE, N=8 for Zk-OBN, Zk-OBN and Zk-LN and N=8 for Zk-LC. Values in the same row not sharing a common superscript (x, y, z) are significantly different ($P \leq 0.05$, ANOVA analyses with LSD post hoc tests). Abbreviations: Zk-OBN – Zucker Obese normal B_6 group; Zk-OBH – Zucker Obese high B_6 group; Zk-LN – AOM-injected Zucker Lean rats; Zk-LC – Control group of Zucker lean rats.

Table C6. Complete blood count[1,2,3] in AOM-injected female Zucker Obese and Lean rats after dietary treatment with vitamin B_6

Parameters	Zk-OBN	Zk-OBH	Zk-LN
WBC (10^9/L)	7.7 ± 1.1^x	5.5 ± 0.8^x	5.2 ± 1.0^x
RBC (10^12/L)	9.2 ± 0.2^x	9.0 ± 0.2^x	8.6 ± 0.1^x
Hb (g/L)	150.3 ± 1.6^x	149.1 ± 2.4^x	NA
HCT (L/L)	0.45 ± 0.01^x	0.45 ± 0.01^x	0.43 ± 0.00^x
MCV (fL)	49.0 ± 0.8^x	49.4 ± 0.5^x	49.3 ± 0.6^x
MCH (pg)	16.4 ± 0.2^x	16.8 ± 0.2^x	17.3 ± 0.2^y
MCHC (g/L)	336.4 ± 3.0^x	334.4 ± 2.1^y	341.7 ± 2.2^x
RDW (%)	14.4 ± 0.2^x	15.0 ± 0.3^x	13.1 ± 0.5^y
Platelets (10^9/L)	975.4 ± 43.3^x	833.5 ± 46.8^y	800.3 ± 59.4^y
MPV (fL)	7.4 ± 0.2^x	7.5 ± 0.1^x	5.9 ± 0.9^y
Seg Neutrophil (10^9/L)	2.4 ± 0.3^x	2.0 ± 0.3^x	1.0 ± 0.2^y
Lymphocyte (10^9/L)	4.7 ± 0.7^x	3.0 ± 0.6^x	3.9 ± 0.9^x
Monocyte (10^9/L)	0.4 ± 0.1^x	0.3 ± 0.0^y	0.1 ± 0.0^y
Eosinophil (10^9/L)	0.08 ± 0.01	0.09 ± 0.01	0.11 ± 0.02

1. Values are expressed as mean ± SE. N=8 for Zk-OBN and Zk-OBH dietary groups and N=6 for Zk-LN.
2. Values in the same row without a common superscript are significantly different ($P<0.05$, ANOVA analyses with LSD post hoc tests).
3. Abbreviations: Zk-OBN, Zucker Obese normal B_6 group; Zk-OBH, Zucker Obese high B_6 group; Zk-LN, AOM-injected Zucker Lean receiving normal B_6; WBC, white blood cell count; RBC, red blood count; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width; MPV, mean platelet volume; NA, not available.

Table C7. Biochemical status of plasma from AOM-injected female Zucker Obese and Lean rats after dietary treatment with vitamin B_6[a]

Biochemical Parameter	Zk-OBN	Zk-OBH	Zk-LN
Total Protein (g/L)	80.0 ± 1.7^{x}	76.4 ± 1.4^{x}	75.0 ± 1.3^{y}
Albumin (g/L)	44.3 ± 2.2^{x}	42.8 ± 1.0^{x}	53.0 ± 1.3^{y}
Globulin (g/L)	37.6 ± 0.8^{x}	33.6 ± 1.4^{y}	22.8 ± 1.3^{z}
A:G Ratio	1.1 ± 0.0^{x}	1.2 ± 0.0^{x}	2.4 ± 0.1^{y}
Urea (mmol/L)	6.7 ± 0.4^{x}	10.3 ± 3.4^{x}	7.4 ± 0.5^{x}
Glucose (mmol/L)	14.8 ± 0.8^{x}	14.9 ± 1.8^{x}	7.6 ± 0.5^{y}
Cholesterol (mmol/L)	11.9 ± 0.4^{x}	9.5 ± 1.1^{y}	2.5 ± 0.2^{z}
Total Bilirubin (umol/L)	3.5 ± 0.3^{x}	6.1 ± 2.8^{x}	2.5 ± 0.2^{x}
Alkaline Phosphatase (U/L)	313.1 ± 46.0^{x}	237.9 ± 23.6^{x}	184 ± 56.6^{y}
Triglycerides (mmol/L)	3.5 ± 0.5^{xy}	5.1 ± 1.4^{y}	1.2 ± 0.3^{x}
CK (umol/L)	562.9 ± 82.1	649.6 ± 118.7	427.7 ± 41.7
Creatinine (umol/L)	43.9 ± 3.7^{x}	39.1 ± 2.3^{x}	44.8 ± 5.2^{x}
ALT (U/L)	126.1 ± 35.1^{x}	127.8 ± 19.9^{x}	36.8 ± 1.4^{y}

[a]Values are expressed as mean ± SE. N=8 for Zk-OBN and Zk-OBH dietary groups and N=6 for Zk-LN. Values in the same row without a common superscript are significantly different ($P<0.05$, ANOVA LSD). Abbreviations: Zk-OBN, Zucker Obese normal B_6 group; Zk-OBH, Zucker Obese high B_6 group; Zk-LN, AOM-injected Zucker Lean group receiving normal B_6, ALT, Alanine aminotransferase; CK, creatine kinase.

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