THE EFFECTS OF CONSUMPTION OF COOKED/CURED MEAT AND GREEN TEA CATECHINS ON XENOBIOTIC METABOLISM AND LONGEVITY IN F344 RATS

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ABSTRACT

THE EFFECTS OF CONSUMPTION OF COOKED/CURED MEAT AND GREEN TEA CATECHINS ON XENOBIOTIC METABOLISM AND LONGEVITY IN F344 RATS

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This thesis presents an investigation of the effect of consumption of cooked/cured meat and green tea catechins on xenobiotic metabolism and chronic disease patterns in Fischer 344 rats. Consumption of cooked and cured meat has been associated with increased risk of gastrointestinal cancer. Consumption of green tea catechins has been associated with decreased risk for gastrointestinal cancers. Cooked/cured meat contains known secondary carcinogens, including heterocyclic amines (HCA), polycyclic aromatic hydrocarbons (PAH) and nitrosamines (NA). HCA, PAH and NA are bioactivated to carcinogens in the body by the cytochrome p450 isoforms CYP1A1/A2 and CYP2E1. Green tea catechins are thought to alter the expression and function of these p450 enzymes, as well as the protective conjugation enzymes, including glutathione S-transferases (GST) and UDP-glucuronyl transferases (UGT). To examine this we explored the effect of: (1) lifetime feeding of hot dog, and hot dog containing green tea on the morbidity and the incidence of gastrointestinal

cancers in F344 rats; (2) chronic consumption of hot dog or hot dog containing green tea catechins on CYP1A1/A2, CYP2E1 and AhR induction; and (3) chronic consumption of green tea catechins or hot dog containing green tea on induction of GST and UGT. Rats fed hot dog and hot dog containing green tea diets had a lower incidence of large granular lymphocytic (LGL) leukemia than rats fed the control diet. In addition, rats fed hot dog and hot dog containing green tea diets did not develop gastrointestinal cancers. Consumption of hot dog resulted in upregulation CYP1A1/A2 and 2E1 in some gastrointestinal tissues and green tea catechins inhibited this upregulation in some tissues. Green tea catechins and hot dog consumption resulted in upregulation in UGT and GST in some gastrointestinal tissues. In conclusion, rats are resistant to intestinal carcinogenesis induced by consumption of cooked/cured meat, and this diet with supplemental catechins may protect against the development of LGL, which is a common cause of mortality in F344 rats.

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LIST OF ABBREVIATIONS

ABC: ATP binding cassette

AAC: amino carbolines

AhR: aryl hydrocarbon receptor

APC: adenomatosis polyposis of the colon

ARE: antioxidant response element

ARNT: AhR nuclear receptor

BaP: benzo[a]pyrene

CAR: constitutive aldosterone receptor

Ccon: combined control group

CON: control diet

CGT: control diet containing 0.5% green tea catechin

CI: confidence interval

DMN: dimethylnitrosamine

EC: epicatechin

ECG: epicatechin gallate

EGC: epigallocatechin

EGCG: epigallocatechin-3-gallate

EGFR: epidermal growth factor receptor

EH: epoxides hydrolase

ER: endoplasmic reticulum

GST: glutathione S-transferase

HCA: Heterocyclic amine

HD: hot dog diet

HDGT: hot dog containing 1% green tea catechin

HNF: hepatocyte nuclear factor

IkB: inactivates NFkB

I.P.: intraperitoneal injection

IQ: imidazo quinolines

Keap 1: Kelch-like ECH related protein

LGL: large granular lymphycytic leukemia

MAPK: mitogen activated protein kinase

MMP: matrix metalloproteases

MeIQx: imidazo quinoxaline

MRP: multidrug resistance protein

NA: nitrosamines

NAT: N acetyl transferase

ngHD: non grilled hot dog diet

NO: nitric oxide

Nrf2: nuclear factor E2 p45

OR: odds ratio

PAH: polycyclic aromatic hydrocarbons

PAPS: phosphoadenosine – 5`- phosphosulfate

Pgp: p-glycoprotein

PH: hot dog diet fed in conjunction with 1% green tea catechin

PHIP: 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (imidazo pyridines)

PI3K: phosphoinositide 3 kinase

PKC: protein kinase C

PXR: pregnane X receptor

RR: relative risk

SLUG: transcription factor related to SNAIL it is not an abbreviation

SMAD4: mothers against decapentaplegic homolog 4

SULT: sulfonyl transferase

TCDD:2,3,7,8 tetrachlorodobenzo-p-dioxin

TP53: tumour protein 53

UGT: UDP-glycuronyl transferase

WCRF: world cancer research fund

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Chapter 1: Review of current literature

Evidence for meat consumption and gastrointestinal cancers

Cancer is the second leading cause of mortality in Canada. Cancers of the gastrointestinal tract (esophagus, stomach, small and large intestine) comprise approximately 17% of all cancer-related deaths. There is strong evidence that cancers related to the gastrointestinal tract are associated with lifestyle factors such as low vegetable consumption and the consumption of cooked, processed or cured red meats. In 1997 the World Cancer Research Fund (WCRF) listed red meat consumption as a dietary risk factor for colorectal cancer(1). Since that time many studies have sought to characterize the exact relationship between gastrointestinal cancer incidence and red meat consumption. It is accepted that it is not necessarily the consumption of red meat alone that is a risk factor for gastrointestinal cancer. The method of cooking and or preserving the meat is a major contributing factor to this type of cancer.

A recent meta-analysis conducted by WCRF (2007) examined the current literature(2). A judgment panel was struck to examine the gathered research and then to conduct a meta-analysis. The results were then categorized according to location of primary cancer. The two main overall recommendations of the report regarding nutrition are to limit the consumption of cooked and processed meat and increase the consumption of fruit and vegetable based foods. Specifically, the report recommends the personal intake of cooked red meat to be less than 500 grams per week and little to none of that should be processed meat. Processed meat is defined as meat that has been smoked, cured or had the addition of chemical preservatives during the processing of the meat. In addition,

the report also provides a recommendation for the increase in consumption of fruits and non-starchy vegetables to at least 400 g/day. In this report, the association of dietary/lifestyle factors with cancer risk are categorized as: convincing, probable, limited suggestive, limited risk/no conclusion and substantial effect on risk unlikely (2).

The panel examined 262 publications on esophageal cancer, concluding that processed meat was in the limited risk category as a risk factor for esophageal cancer. Fruits, non-starchy vegetables are reported as probable in their ability to reduce the risk of esophageal cancer (2). The evidence from 722 publications on stomach cancer was examined. From these publications the WCRF has listed the consumption of non-starchy vegetables and fruits as probable in their ability to decrease the risk of stomach cancer. Processed, smoked, grilled or barbequed animal based protein is categorized as limited suggestive in its ability to act as a risk factor for stomach cancer. The judgment panel of the WCRF examined the results of 752 publications on colorectal cancer. The evidence from the analysis of these publications resulted in the WCRF listing the consumption of red meat and processed meat as convincing risk factors for colorectal cancer. In addition, the consumption of non starchy vegetables and fruit are listed as limited suggestive in their ability to decrease the risk of colorectal cancer (2). The following sections will expand on the risks associated with red meat consumption, including degree of cooking and preservation methods.

Association of meat consumption, preparation and preservation related to incidence of gastrointestinal tract cancer: epidemiological evidence

Esophageal cancer

As mentioned above, the WCRF recently published a meta-analysis of all published literature relevant to dietary or life style causes of cancer. The association between red meat consumption and esophageal cancer was assessed with the analysis of 12 case control studies. These studies controlled for smoking, which is a major risk factor for esophageal cancer. When the highest level of meat consumption was compared to lowest level of meat consumption eight of these studies found an increased risk of developing esophageal cancer associated with red meat consumption. Five of these studies reported a significant increase in esophageal cancer risk when the highest intake level of red meat was compared to the lowest intake level. The WCRF report does not indicate how intake level was classified possibly because they are examining different studies that use different intake classifications. The remaining three studies found no increase in risk with increasing red meat consumption(2). There is limited evidence that associates risk of developing esophageal cancer with consumption of red meat.

Stomach cancer

In the WCRF report the consumption of red meat has not been associated with stomach cancer risk. The main association of stomach cancer and meat comes with the consumption of processed meat (2).

Colorectal cancer

In the WCRF report, 16 cohort studies and 71 case control studies were used to examine the link between red meat consumption and colorectal cancer. In the cohort studies the risk of developing colorectal cancer was assessed by comparing cancer incidence in the highest red meat intake group to the lowest intake. The studies that reported red meat intake in times per week were included in the meta-analysis. The summary effect for this meta-analysis was 1.43 (95% confidence interval (CI) 1.04-1.94) with increasing consumption in times per week. The estimated risk for increasing meat consumption by 100g/day was 1.29 (95% CI 1.04-1.60) (2).

The results of the WCRF report are supported by a more recent meta-analysis of 103 epidemiological studies conducted from 1966 to 2008. The meta-analysis examined the relationship between cooked meat consumption and colorectal cancer risk. They reported a relative risk of 1.21 (95%CI of 1.13-1.29) when individuals ingesting meat in the highest tertile of consumption were compared to individuals in the lowest tertile of cooked meat consumption (2;3).

Impact of cooking methods and doneness

Red meat is generally consumed cooked and/or cured/ processed. Cooking of meat reduces potential pathogen exposure and enhances flavor. However, epidemiological evidence indicates that cooking can increase the carcinogenic potential of meat. Cooking methods include grilling, pan frying and roasting,

which generate different temperatures. All of these methods can be conducted for various durations leading to different levels of doneness that lead to formation of polycyclic aromatic hydrocarbons (PAH) and heterocyclic amines (HCA). Some older studies have produced equivocal results; Knekt and coworkers did not find risk associated with pan fried meat consumption in 1994 (4). Iscovich et al. (1992), found that consumption of fried, grilled or baked meat was not associated with an increased risk of colorectal cancer(5). The data appear to be changing in more modern literature, perhaps due to more effective food surveys or the fact that more people are consuming less-cooked meat, creating a range of doneness levels for analysis.

Sinha and coworkers studied the effect of cooking duration in a case control study, using 146 patients with colorectal cancer and 226 controls. Risk was calculated by comparison of the highest and lowest quintile of cooked meat consumption. The OR for increased cancer risk associated with all sources of red meat was 1.11 (95% CI 1.03-1.19) per 10 g intake per day(6). However, the risk increased dramatically, to 1.29 per 10 g intake per day, for well done/very well done red meat. Matos and Brandani (2002) reported that the risk for gastric cancer was significantly elevated with the consumption of fried meat in a South American population(7). Gunter et al. (2005) estimated HCA and benzo[a]pyrene (BaP) exposure from food records and found a significant risk associated with BaP exposure, and barbequing of foods, with an OR of 1.90 for colorectal adenoma in the top quintile of barbequed red meat intake(8). Sinha et al. (2005)

analyzed close to 4000 adenoma cases and 35,000 controls, finding an association of adenoma incidence with well done red meat consumption, and BaP and HCA exposure(9). In 2007, Shin et al. reported that the estimated exposure to HCA from cooked red meat was associated with the risk of developing colorectal polyps(10). Ferrucci et al (2009) added to these data by surveying a large population of asymptomatic women who were about to be screened by colonoscopy. In this strong design, which limited recall bias, the risk of having a colorectal adenoma was associated with red meat consumption, pan-fried meat consumption and HCA exposure(11).

In addition to the risk associated with cooked red meat, colorectal cancer risk is also associated with the consumption of processed meat. Processed meat constitutes approximately one half the red meat consumption in the world(12). The most common method of processing meat is via curing, followed by smoking. Curing of meat products like sausages, hot dogs and bologna can be readily accomplished by mixing nitrate or nitrite salts into the ground meat before packing. Solid meat products, like ham, need to be soaked in salt/nitrite solutions for extended periods. Curing prevents bacterial growth and provides a distinctive taste(12). The bright red colour in cured meats is due to the formation of nitric oxide which then binds to the iron in hemoglobin keeping it in a reduced state. Curing is usually followed by smoking, where the meat is hung in the presence of smoldering wood chips and the meat is allowed to cook slowly at between 155 - 200°C while absorbing the lipophyllic smoke constituents, including PAH(12).

Processed meat and esophageal cancer

The data on the association between processed meat consumption and esophageal cancer are limited. The WCRF report examines 2 cohort and 8 case control studies. Of the eight case control studies 6 of them reported a higher risk in esophageal cancer in the highest intake group when it was compared to the lowest intake level of processed meat, but only one of these was statistically significant. The 2 cohort studies adjusted for age, smoking and alcohol intake and reported a non-significant increase in risk when the highest level of processed meat was compared to the lowest intake level(2). More recently, Aune et al. (2009) reported the risks of developing various cancers in Uruguay, finding that processed meat intake was associated with increased risk of developing cancers of the esophagus, larynx, stomach, colon and lung (13).

Processed meat and stomach cancer

The WCRF (2007) reports on 8 cohort studies and 21 case control studies examining the risk of stomach cancer associated with consumption of processed meat. Only one of the cohort studies reported a significant increase in stomach cancer with processed meat consumption and the meta-analysis resulted in a non-significant summary effect estimate of 1.02 (95% CI 1.0-1.05) for 20g/day intake of processed meat. The evidence from the case control studies suggested a dose response relationship between processed meat and risk of stomach cancer. The meta-analysis conducted on nine studies resulting in a summary effect estimate of 1.13 (95% CI 1.01-1.25) for 20g/day intake of processed

meat(2). It is possible that the significance in case control studies reflects recall or selection biases. To summarize, there appears to be a limited association between stomach cancer and processed meat consumption, however the evidence appears contradictory.

Huxley et al. (2009) reported the results of a meta-analysis of 103 epidemiological studies associating the consumption of processed meat with the risk of developing colorectal cancer. (RR = 1.19; 95% CI 1.12-1.27)(3). In another review of case control epidemiological studies, Norat et al. (2001) reviewed studies on processed meat and colorectal cancer, reporting significant odds ratios ranging from 1.18 to 2.9 (14). A subsequent article published by Norat et al (2002) conducted a dose response analysis for processed meat consumption. When the consumption of 30g/day of processed meat was compared to no processed meat the relative risk was 1.32 (95% CI 1.02-1.70) for colon cancer(15). The WCRF report (2007) examined 14 cohort studies and 44 case control studies on colorectal cancer and processed meat. Twelve studies found higher risk, but only 3 of these were significant. A meta-analysis was conducted on 5 studies that had comparable category definitions, resulting in a summary effect estimate of 1.21 (95% CI 1.04-1.42) for 50g/day of processed meat consumption(2). In the last several decades there have been a large number of both cohort and case control studies that have examined the effect of processed meat consumption on colorectal cancer risk. The bulk of this evidence suggests that increasing consumption of processed meat increases the risk of developing colorectal cancer, but the effect is not dramatic and many of

the studies are not of high quality. When comparing survey data on red meat versus processed meat (which is also mainly red meat), there does not appear to be any difference in risk for developing gastrointestinal cancers. However, given the proposed mechanisms of how meat causes gastrointestinal tract cancers smoking and curing should enhance the carcinogenic potential of red meat. There are 3 major factors that can affect the outcome of epidemiological studies. Epidemiological studies rely heavily on the memory and accurate recall of the participants. They frequently do not account for fruit and vegetable consumption of the participants. Lastly they often fail to stratify the responses according to temperature and duration of cooking method for the meat consumed. While survey-based studies can never prove causality, satisfying Hill's Criteria can interpret the strength of the data and help to infer causality. Meta-analyses demonstrate some consistency between studies, and there is some evidence of dose response. There is not great strength in the size of the risk or odds ratios, although even small risks for common disease can represent important problems. The important Hill's Criteria of biological plausibility is usually generated through well controlled experiments using in vitro or animal-based models. The mutagenicity and carcinogenicity of purified PAH, HCA and NA have been demonstrated in such models, but there is a lack of data on the ability of cooked/preserved meat to cause cancer in controlled animal experiments.

Theories related to the causative relationship between meat and gastrointestinal tract cancer

There are several published theories that explore the mechanisms by which cooked, cured or preserved meat could cause gastrointestinal tract cancers. Red meat is relatively high in saturated fat, which stimulates increased bile acid production. This increase in bile acids in the distal colon is thought to be tumour promoting (16;16;17). In addition red meat also contains more iron than white meat. High dietary iron is poorly absorbed and will lead to high fecal iron exposed to the colonic mucosa, potentially causing oxidant stress and a variety of other metabolic effects. High consumption of heme based iron increases the development of dimethylhydrazine-induced colorectal tumours in mice and rats (18). Other theories relate to the levels of heterocyclic amines (HCA), polycyclic aromatic hydrocarbons (PAH) and nitrosamines (NA) in cooked and cured meats. HCA, PAH and NA are lipophyllic secondary carcinogens that are present in cooked and preserved meats such as sausages, hot dogs and smoked cold cut meats (19). Once ingested these molecules are metabolized to their carcinogenic forms by a process called bioactivation or biotransformation. For the purposes of this literature review we will be concentrating on the evidence surrounding the consumption of meat containing HCA, PAH and NA, and their relationship to gastrointestinal tract cancer. HCA, PAH and NA are chemicals that are produced during the process of cooking and curing meat and are metabolized as xenobiotics by the body.

Evidence linking consumption of BaP, PHIP and DMN to gastrointestinal tract cancer

Dietary xenobiotics must pass through the gastrointestinal epithelium before entering the hepatic portal circulation, or being released into the lymphatic flow as a chylomicron. Both of these routes will bring the xenobiotic to the liver, where the capacity for phase I and II metabolism is dramatically higher than that of the gastrointestinal tract and other tissues. To the extent that the gastrointestinal tract does express P450 enzymes, some portion of dietary secondary carcinogens may be bioactivated before reaching the liver. This makes gastrointestinal tract tissues potential targets for cancers induced by dietary sources of HCA, PAH and NA.

Heterocyclic aromatic amines (HCA)

HCA are formed when meat is cooked at temperatures over 100°C, although the reactions are faster as the temperatures increase above 200°C. These reactions are favored during drying of the meat or protein containing residue (e.g. gravy). During this process creatine is converted to creatinine which undergoes reactions with amino acids like phenylalanine, threonine, or alanine to form HCAs. Sugars may increase the reaction rate or change the end products but are not essential for HCA formation. Cooked red meat HCA content varied from 0.1 to 14 ng/g in samples obtained from restaurants(20). Higher cooking temperatures and longer

duration of cooking can greatly increase the HCA levels, especially as the meat dries out, increasing the internal temperature of the meat and the effective concentration of reactive substrates.

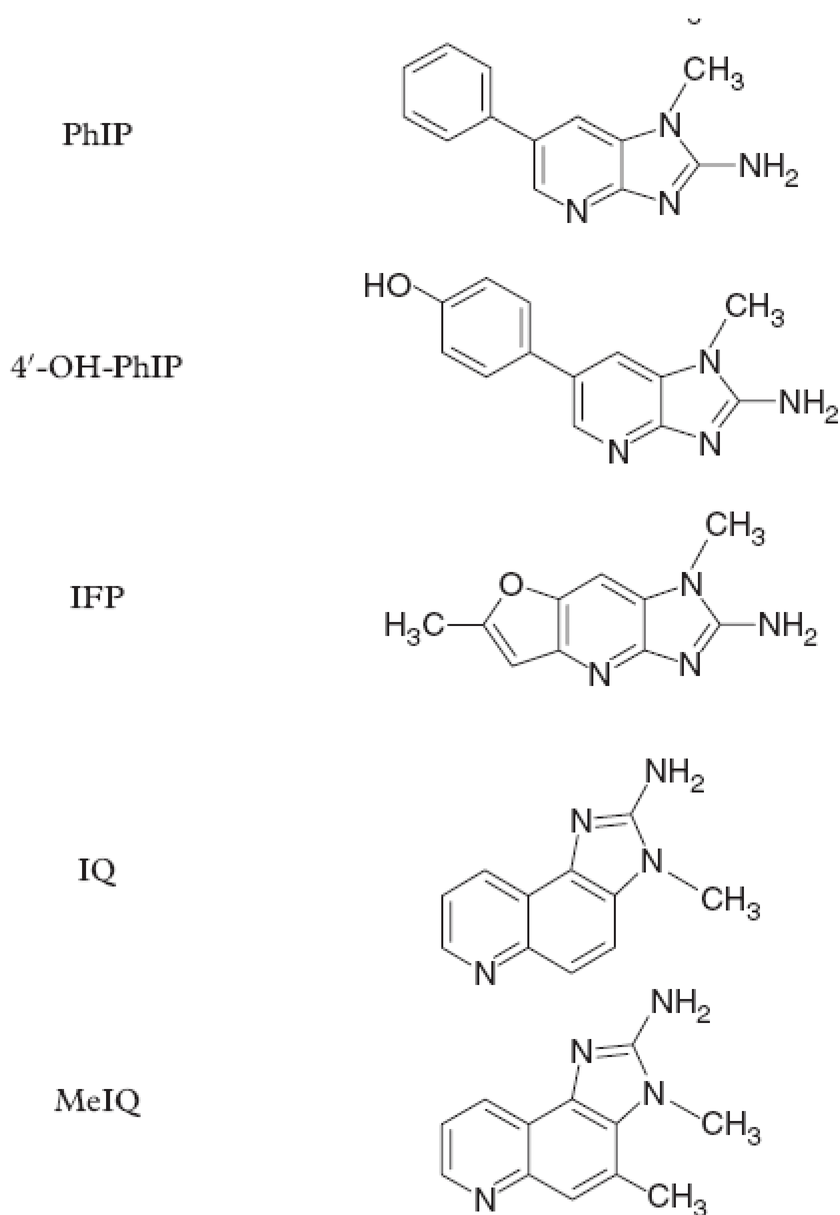

Figure 1: Example of heterocyclic amines present in cooked meat(21).

HCAs are a class of 17 small, relatively nonpolar molecules with exocyclic amino groups and several nitrogen atoms(20) (figure 1). There are 4 main classes of HCA; AAC (e.g. amino carbolines), imidazo-quinolines (e.g. IQ), imidazoquinoxalines (e.g. MeIQx) and imidazopyridines (e.g. PhIP). These HCAs are secondary mutagens that have been shown to cause cancer in liver, skin, lung, colon and mammary gland in mice, rats and some primates (15). PhIP is the most abundant HCA found in cooked meat. Ingestion of PhIP has been associated with colorectal cancer risk in human populations(22). The bioavailability of PhIP was determined in Wistar rats to be approximately 0.79% following gavage with labeled [C^{14}] PHIP with peak blood levels attained 30 minutes after gavage. This blood level declined steadily over 48 hours to approximately 0.26% (23). In addition, when 70-84 ug of PhIP was given to humans undergoing colonic tumour surgery, 42-122 pg PhIP was present per gram of colonic tissue (24). Consumption of PhIP has been implicated in a variety of types of cancers including mammary gland, large intestine, prostate and lymphoid tissue cancers in monkeys, rats and mice (25). Baranczewski et al (2004) treated mice by gavaged with PhIP for three days, leading to the accumulation of DNA adducts in the livers of these mice(26). F344 rats were fed a diet containing 4ppm PhIP for 43 weeks, leading to the development of well differentiated large intestinal adenocarcinomas in the colons of 45% of the rats (27). The carcinogenic characteristics of PhIP were also seen in rats fed a high fat diet that contained PhIP. In this experiment male F344 rats were fed a diet containing 400ppm PhIP for 3 two week intervals over the course of 14 weeks in

conjunction with a high fat diet (59% of calories coming from hydrogenated vegetable oil). Rats were then fed a high fat control for the remaining experimental period. Large intestinal adenocarcinomas were found in 45% of the rats. A similar experiment using B57BL/N6 mice fed a high fat diet (23% hydrogenated vegetable oil) containing 300ppm for 40 weeks found that 52% of male and 68% of female mice had small intestinal adenocarcinomas and adenomas. In addition this experiment examined the incidence of PhIP induced B-cell derived lymphomas. Forty eight percent of male mice and 32% of female mice had B cell lymphomas. Interestingly PhIP also induced an earlier onset of lymphoma in female mice (28).

Other HCA, like IQ and MeIQx, are present in cooked meat together with PHIP. They are present in smaller quantities when compared to PHIP but they are believed to be more carcinogenic. When a diet containing 400ppm MeIQx was fed to F344 rats for 61 weeks the animals developed multiple cancers in liver, zymbal gland, clitoral gland and skin (29). Similar results were seen in C57BL/6 mice fed a diet containing 600ppm of MeIQx. Following 84 weeks of feeding the mice developed tumours in the liver, lung and hematopoietic system (30).

Ohgaki et al in 1984 investigated the effect of IQ on CDF mice. The mice were fed a diet containing 300ppm for 96 weeks. By the end of the experimental period the mice had developed liver, foreskin and lung tumours(31).

Polycyclic aromatic hydrocarbons (PAH)

Polycyclic aromatic hydrocarbons are created when foods are cooked at carbonization temperatures (>250°C). PAH are created following partial pyrolysis of essentially any food substrate. The combustion process leads to the formation of 2 and 4 carbon radicals that, if combustion is incomplete, will recombine to form the stable PAH ring structures. PAH formation is usually greater in high fat foods due to the volatilization and combustion of fats during cooking. During the grilling process fat from meat drips onto the hot surface resulting in flames, which then deposit PAH on the surface of the food (22;32). The most common and best characterized food borne PAH is benzo[a]pyrene (BaP) (figure 2). PAHs contain three or more fused benzene rings with no acyclic groups.

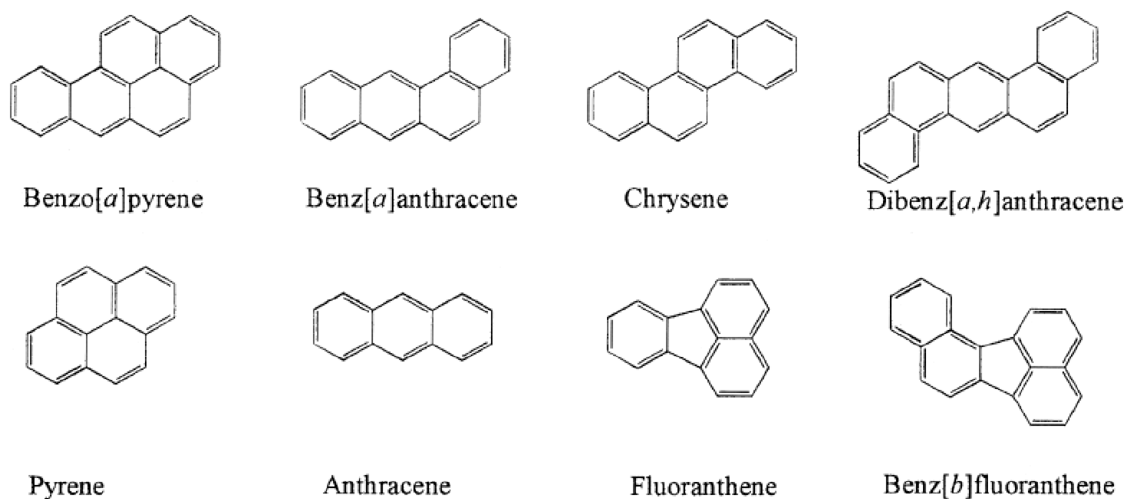

Figure 2: Polycyclic aromatic hydrocarbons found in the environment and present in food(33).

On average, grilled meat contains approximately 10.5ng/g of BaP. The reported dose (either ingested or subcutaneous injection) of BaP required to elicit a

cytotoxic response in the body is 50-100mg/kg body weight. The average mature male human ingests approximately 3.12 mg of PAH/day with diet contributing 92% of this amount (34). Other sources of PAH may include tobacco smoke, chewing tobacco, and car exhaust. The bioavailability of [C^{14}] labeled BaP was estimated in pigs to be around 33% (35). Once in the mucosal cells the ATP binding cassette (ABC) transport proteins may transport BaP back into the lumen of the small intestine(36). Helleberg et al (2001) examined the effect of BaP intragastric exposure on mice. The mice used in this experiment were a cross of Xpa deficient and p53 heterozygous transgenic mice crossed with C57Bl/6 mice. The mice were gavaged with 13 ppm BaP three times a week for seven weeks. Mice were sacrificed and the number of DNA adducts was examined in liver, lung, spleen, forestomach, stomach, small intestine, and colon. Interestingly the liver and lung had the greatest number of adducts. Gastrointestinal tract tissues had approximately half the number of DNA adducts when compared to the liver thus indicating that liver may be more susceptible to DNA damage than gastrointestinal tract tissues(37). This is most likely because of the greater capacity for bioactivation present in the liver.

Nitrosamines (NA)

There are several possible mechanisms for the formation of nitrosamines in foods and in the gastrointestinal tract. The formation of nitrosamines results from a reaction between nitrite and a secondary or tertiary amine. Much of the total dietary nitrate is present in fruits and vegetables; plants accumulate nitrate, and this is enhanced by fertilizing. Fortunately, fruits and vegetables have a low

concentration of amines and a high content of reducing agents, which both limit the formation of NA. Because of this, total dietary nitrate doesn't correlate well with nitrosamine exposure.

Intake of red meat leads to increased availability of amino acids, resulting in decarboxylation to form amines and amides which are then converted to NA when nitrite is present. When nitrite is consumed in the diet in the form of sodium nitrite added for curing purposes, it will be available to react with amines to form NA. Nitrate from fruits and vegetables will be less likely to participate in the formation of NA due to presence of reducing agents, which convert nitrite to nitric oxide,

Sodium nitrite is used to prolong shelf life of meat and limit the risk of bacterial growth. Nitrate is present in a variety of foods including: vegetables (approximately 1-2 mg/kg), dairy products (approximately 5mg/kg), beer (approximately 16 mg/kg), cereals and grains (approximately 12 mg/kg) drinking water (does not exceed 50mg/liter). Both nitrate and nitrite may be present in cooked and cured meat at approximately 26 – 43 mg/kg depending on the country of origin's regulations. In Canada, the total amount of nitrate and nitrite is limited, causing food producers to only use the more potent preservative, nitrite.

Nitrate can be reduced by bacterial nitrate reductase to form nitrite in the mouth and distal small intestine, and low pH can reduce nitrate to nitrite in the

stomach(38). Consequently, both nitrate and nitrite can be converted to DMN in the body (figure 3).

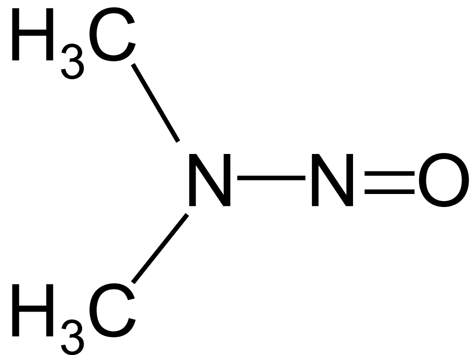

Figure 3: Structure of dimethylnitrosamine.

In a study conducted by Luca et al. (1985), mice were dosed with one of the following levels of sodium nitrate; 78.5, 236, 707, or 2120 mg/kg body weight. Mice received two doses of sodium nitrate 24 hours apart. Mice were sacrificed and bone marrow was isolated and examined for micronuclei. The middle two doses corresponded with a significant increase in micronuclei in bone marrow. The same experiment conducted on rats and found no effect on the bone marrow(39). A two year study was conducted feeding rats a diet containing 40% canned meat which contained either 0.02% or 0.5% sodium nitrite. There was no significant increase in incidence of tumours or pre neoplastic lesions between control and treatment animals(40).

The experimental evidence outlined in the sections above illustrates the carcinogenic quality of each of the main carcinogens present in cooked and

cured meat. One potential criticism of this research is that it only establishes the tumour forming potential for each of the individual xenobiotics. This is not how the carcinogens are presented to the body in food, where the carcinogens are mixed, and present at very low quantities. In a food matrix, carcinogens may have different bioavailability than in purified powdered diets, and both of these will be likely be lower than gavaged suspensions or injections.

Biological response to a xenobiotic encounter

The potential relationship between HCA, PAH and NA and gastrointestinal tract cancers is related to how the body metabolizes these xenobiotics. Xenobiotic metabolizing enzymes oxidize and conjugate HCA, PAH and NA producing reactive intermediates that are potential carcinogens. The following sections examine xenobiotic metabolism of HCA, PAH and NA.

Foreign chemicals, or xenobiotics, can be harmful, benign or helpful. Xenobiotic metabolism requires a large number of enzymes with overlapping specificity that are responsible for protecting the body against accumulation of foreign chemicals. Many of these enzymes have low substrate specificity and efficiency but they exist in high concentration within cells and there is also a high degree of redundancy between the various enzymes. They may be regulated by transcriptional, post-transcriptional and post-translational mechanisms; in times of high xenobiotic exposure there is often a corresponding upregulation of the required enzymes.

There are various possible routes of exposure to xenobiotics. They may be ingested, absorbed through the skin, inhaled, or injected. Metabolized

xenobiotics can leave the body via feces, urine (the most common routes), sweat or exhalation. The most common routes of excretion are in the bile and urine. Biliary excretion is effective for lipophilic molecules, but can result in enterohepatic circulation. Urinary excretion is a more certain route of elimination, but it requires greater water solubility. Lipophyllic molecules such as PAH, HCA and NA can be made more water soluble by conjugation to a soluble substrate (40;41).

Xenobiotic metabolism consists of different phases, which can work in series, or independently, depending on the xenobiotic metabolized. Phase 1 involves cytochrome p450 enzymes which are microsomal enzymes, also referred to as monooxygenases or the mixed function oxidase system. Glutathione S-transferases (GST), UDP-glucuronal transferases (UGT), sulfonyl transferases (SULT) and N acetyl transferases (NAT) are phase 2 conjugation enzymes, which are either cytosolic or bound to the endoplasmic reticulum (also called microsomal enzymes). Phase 3 involves transport by the multidrug resistance proteins (MRP) and p-glycoprotein (Pgp). For the purposes of this review we will be concentrating our discussion on Phase 1 and Phase 2 enzymes (41;42).

Phase 1 xenobiotic metabolism: CYP450

Cytochrome p450 (CYP) is a super family of monooxygenase enzymes that have a variety of important roles in the body, including hormone synthesis, heme and bile synthesis and xenobiotic metabolism. They are located in the endoplasmic reticulum of most cells in the body. In mammals the CYP super family has a

similar core of around 12 families and 22 subfamilies. Theses enzymes are grouped together based on similarity of amino acid sequences. In humans there are 18 families, and 43 subfamilies with 57 known genes and numerous pseudogenes. There are four CYP gene families that handle the bulk of xenobiotic metabolism, namely CYP1-4. Each of these families contains multiple subfamilies. However, CYP1A1, 1A2 and 2E1 are most involved in the metabolism of PAH, HCA, NA and EGCG (43). Not all P450 enzymes are expressed in all tissues. For example, CYP1A1 may be expressed in lung, esophagus, stomach, small intestine and large intestine, where it plays a role in the bioactivation of PAH and HCA. CYP2E1 may be expressed in the lung, esophagus, and small intestine, where it can play a role in NA bioactivation (44;45).

Xenobiotic metabolism: CYP upregulation

Many P450 genes are upregulated in response to xenobiotic exposure. Xenobiotics can be anything from beneficial phytochemicals to cytotoxic chemotherapy drugs. PAH, HCA and NA are lipophyllic molecules that diffuse through the cell membrane. Once in the cell some portion of the xenobiotic molecules will bind to P450 isozymes to be metabolized, while some may bind to various cytosolic receptors, which will trigger the transcription and translation of additional CYP proteins and other phase 2 enzymes.

Nuclear receptors involved in PAH, HCA and NA metabolism

There are four main cytosolic receptors that are involved in HCA, PAH and NA bioactivation and green tea catechin metabolism. The aryl hydrocarbon receptor (AHR) is a cytosolic receptor present in lung, thymus, kidney, and liver and it is involved in the upregulation of CYP1A1, 1A2, UGT and GST (43;46). In its inactive form it is bound to cytosolic chaperone proteins. When a ligand (PAH or HCA) comes into the cell and binds to the AHR-chaperone protein complex the chaperone proteins dissociate and the complex translocates to the nucleus, where AHR nuclear translocator protein (ARNT) binds to AHR-ligand complex. This complex then binds to the AHR response element AHRes located in the promoter region of the gene encoding CYP450 (47;48).

The PXR receptor (pregnane X receptor) is known to be located in the liver, small intestine, colon and in lymphocytes. Similarly to Nrf2 and AHR, in response to xenobiotic stress PXR heterodimerizes with retinoid X receptor and then translocates to the nucleus where it binds to the promoter region for a number of xenobiotic metabolizing enzymes including CYP1A2, UGT and AHR (46;49). Hepatocyte nuclear factor receptor1 (HNF) is present in the liver, pancreas and kidney. In response to xenobiotic stress HNF1 translocates to the nucleus and binds the constitutive androstane receptor (CAR) is expressed in the liver and small intestinal epithelium and is involved in the upregulation of UGT 1A1(46;50). CAR plays an important role in bile acid detoxification. Cooked and cured meat is relatively high in saturated fat, which stimulates increased bile acid production. This increase in bile acids in the distal colon is thought to be tumour promoting

(16;17). Consequently, although there is no direct evidence of CAR being upregulated in the presence of cooked and cured meat it is possible that there is an indirect effect of meat consumption on CAR function.

In addition to control by protein receptors that bind xenobiotics, gene expression can be regulated by sensors of oxidant or electrophilic stress. Nuclear factor E2 p45 related factor 2 (Nrf2) is ubiquitously present in the body. Nrf2 binds to Kelch-like ECH related protein (Keap1) which contains a number of serine and threonine residues. When these residues are phosphorylated by either PKC, PI3K or MAPK (protein kinase C, phosphoinositol 3 kinase or mitogen activated protein kinase respectively) in response to oxidative or electrophilic stress Nrf2-ligand complex is translocated the nucleus where it binds to the promoter ARE (antioxidant response element). ARE then upregulates CYP1A1, 1A2, GST and UGT (46;51;52).

Phase 2 xenobiotic metabolism: conjugation reactions

During phase 2 xenobiotic metabolism, functionalized and often reactive intermediates are conjugated with various substrates depending on their chemical nature and their recognition by suitable enzymes. There are five main families of enzymes involved in phase 2 conjugation reactions, although there are a number of additional conjugation reactions they are not relevant to this study. UDP-glucuronyl transferases (UGT) are a family of microsomal enzymes conjugating glucuronic acid to nucleophilic xenobiotics or metabolites. Glutathione S-transferases (GST) are a family of cytosolic and microsomal enzymes involved in conjugating glutathione to electrophilic molecules. These

two families of enzymes handle a large proportion of xenobiotic conjugation reactions. Sulfonyltransferase (SULT) is a family of mainly cytosolic enzymes which are also involved in conjugating nucleophiles, using 3`-phosphoadenosine-5`-phosphosulfate (PAPS) as a substrate. Methyltransferases are mainly cytosolic and act to methylate substrates using s-adenosylmethionine as a conjugating agent. N-acetyl transferases (NAT) are cytosolic enzymes which acetylate substrates using acetyl-CoA as a conjugating agent. When considering the carcinogenicity of HCA, PAH, and NA, the main focus is on UGT, GST and NAT activities. NAT actually catalyzes the final step in bioactivation of HCA and thus it has a pro-mutagenic effect when HCA are present. The majority of the protection against reactive metabolites of these carcinogens will be provided by GST and UGT enzymes, so the remaining portion of this review will concentrate on these (41).

UDP Glucuronyl transferase (UGT)

Conjugation of a lipophyllic compound to a sugar derivative can increase the solubility of the compound and decrease its reactivity. Glucuronide conjugation is the most active of the phase 2 pathways, and is important in protection against reactive intermediates during metabolism of both PAH and HCA. There are 2 major families of UGT enzymes namely UGT 1 (induced by chlorinated compounds; nitrophenol, halogenated phenols, and bilirubin) and UGT2 (constitutively expressed but also be induced by phenobarbital). Both are bound to the endoplasmic reticulum (ER). Conjugation by UGT occurs in the lumen of

the ER. UDP-glucuronic acid (UDPGA) and the xenobiotic are both located in the cytosol. The products of this reaction are then exported from the lumen of the ER to cytosol(41).

Glutathione S-transferase

Glutathione (GSH) (γ-glutamyl-cysteinyl-glycine) is present in concentrations as high as 10 mM in cells such as hepatocytes. The gamma linkage joining glutamate and cysteine prevents peptidases from rapidly degrading the molecule. Glutathione is the major intracellular reductant and a conjugating agent for electrophilic substrates. Following conjugation, the glutamate and glycine residues are removed forming a cysteine conjugate, which may be excreted or N-acetylated to form a mercapturic acid conjugate, which is excreted in the urine(41).

GST comprises about 5% of human hepatocyte cytosolic protein. There are 7 soluble and 3 microsomal forms of the enzyme in humans, with the expression patterns dependant on the type of tissue (53). The expression of some GST genes is induced by the presence of substrates such as phenobarbital, BaP and TCDD. GST genes can be induced by the interaction of planar molecules with the AHR, or by the impact of electrophiles or reactive oxygen species on the Nrf2/ARE system. Activation of the AHR actually works to prime the ARE system by inducing Nrf2 expression. Certain forms of GST are inducible by the presence of endobiotics like testosterone or estrogen. Declining sex hormone levels with age could explain age related changes in xenobiotic metabolism (41).

Xenobiotic metabolism of HCA, PAH and NA

HCA, PAH and NA are chemicals that are produced during the process of cooking and curing meat and are metabolized to form reactive intermediates by CYP enzymes, including CYP1A1, 1A2 and 2E1. The phase 2 conjugation enzymes (GST and UGT), then conjugate the reactive intermediates to increase their solubility and to facilitate their removal from the body. The following sections expand the bioactivation of HCA, PAH and NA and their subsequent conjugation.

Metabolism of HCA

The bioactivation of HCA is initiated by CYP1A1/2 catalyzed oxidation of the exocyclic amine group of HCA into a reactive hydroxylamine derivative (54). This derivative is O-acetylated by NAT2 creating an N-acetoxy derivative which then undergoes heterocyclic cleavage creating a nitrenuim ion (22;55). The nitrenuim ion is reactive towards DNA and binds to the N2 and C8 atoms of guanine creating adducts. PHIP and IQ-induced genotoxicity in rats is brought about by adduct formation in oncogenes and tumour suppressor genes, where C→T transversons and base deletions lead to permanent mutations. HCA have been shown to induce tumours in a variety of locations in the rat and mouse including liver, lung, small and large intestine (25;55). As mentioned above, phase 2 metabolism is also involved in bioactivating HCA. The pathway involving NAT is

described above, but SULT metabolism of N-hydroxy derivatives can also form ultimate carcinogens from HCA(56).

In contrast, GST and UGT enzymes will help to metabolize HCA intermediates to safe conjugates and prevent carcinogenesis. N-hydroxy derivatives are electrophilic and thus GST can conjugate a glutathione to the derivative thereby facilitating its removal by urine or feces. UGT can conjugate either the exocyclic or imidazole nitrogen atoms of the N-hydroxy derivative making it water soluble and allowing for excretion(54).

Metabolism of PAH

BaP is first metabolized by CYP 1A and CYP 1B to form an epoxide in the 7,8 position. This epoxide is hydrolyzed by epoxide hydrolase (EH) forming a 7,8-diol. Unfortunately, the 7,8-diol is further metabolized by CYP1A1/2 to a 7,8-diol-9,10-epoxide, which is the ultimate carcinogen. BaP diol-epoxides are likely to react with nucleophilic sites on DNA forming adducts, which cause mutations and cancer. Potential DNA adducts resulting from BaP metabolism include N^2-deoxyguanosine, N^7-deoxyguanosine and N^6-deoxyadenine. BaP has been reported to cause G→T transversions in the 12th codon of the Ras family of proto-oncogenes(57). Ingestion of PAH has been shown to induce squamous cell carcinoma in the stomach, lymphoma and leukemia in rats and colon cancer in humans (22;34). The majority of BaP is metabolized by P450, GST and UGT in the liver. Recently, intestinal xenobiotic metabolism has been suggested to play a greater role than previously anticipated. Little is known about BaP

metabolism in the esophagus and stomach. Much of the research associated with esophageal tumours caused by PAH exposure is related to inhaled PAH that gets in to the gastrointestinal tract via the epiglottis (58). In the past, it was thought that food containing BaP passed through the esophagus too quickly to significantly expose this tissue, but PAH are often concentrated on the food surface, and these lipophyllic xenobiotics can pass through the lipid bilayer of the epithelium and potentially become bioactivated by CYP1A1 in the esophagus. Food containing BaP spends a considerable time in the stomach and small intestine, so these tissues will have an opportunity to absorb and metabolize BaP and other PAH. The small intestine may participate in first pass metabolism of BaP, depending on the expression levels of CYP1A1. In mice, orally administered BaP resulted in CYP1A1 induction in small intestinal villus cells. This induction declined towards the ileum. The induction in the small intestine in response to orally administered BaP was much smaller and slower that a comparable dose of BaP administered by i.p. injection (59). This slow induction and unequal distribution of CYP in the small intestine could be a reason as to why there are few small intestinal neoplasm reported in comparison to colorectal cancers. In addition, the lifespan of a small intestine enterocyte is small and thus any induced and bioactivated BaP will be sloughed off and excreted through the gastrointestinal tract (34). Consequently the cancer risk is in the stem cell pool, which might not express much P450. Enterocytes are terminally differentiated, and if they incur DNA damage, they will be sloughed off rather than cause cancer. The large intestine may be exposed to BaP via the blood stream,

although much of it will have been metabolized by enzymes in the small intestine and or liver. However, previously conjugated BaP that is passes back to the large intestine through bile, sloughed cells or MRP action can be can be hydrolyzed and potentially bioactivated by microbial CYPs present in large intestine (34). Also, as mentioned earlier, BaP and related molecules tend to be pumped back into the intestine by MRP leading to low systemic bioavailability, but a large potential exposure to the colonic mucosa.

Metabolism of nitrosamines

Like HCA and PAH, NA are non carcinogenic until they are metabolized by P450 enzymes. Nitrite and amines react to form nitrosamines in foods or in the contents of the gastrointestinal tract. If the reacting amine has methyl group side chains, dimethylnitrosamine (DMN) will be the specific nitrosamine that is formed. DMN can rapidly be absorbed across the duodenal mucosa (38).

DMN is bioactivated by CYP2E1 in the esophagus, small intestine or liver and or 2A6 (in esophagus) creating an electrophilic monoalkylnitrosamine and an aldehyde (45;60;61) . At this point GST may conjugate the monoalkylnitrosamine to a glutathione molecule, making it stable and water soluble. If GST does not conjugate this reactive intermediate then it will rearrange and release a carbocation (carbanion). This is the ultimate carcinogen that can react with DNA bases creating various methylated bases, including 0^6methylguanine. O^6methylguanine is responsible for GC-AT transitions in DNA creating a variety of DNA mutations. The carcinogenic intermediates of nitrosamine bioactivation

are highly unstable so it is most likely bioactivated in the tissues where the tumours develop. However, it is possible for DMN to be bioactivated in one organ and produce a DNA lesion in another tissue(19).

From adduct formation to malignant neoplasm

Once a DNA adduct is formed, it may be repaired via excision repair or it can lead to mutations and potentially initiate the neoplastic process. If the mutation results in oncogene activation or the disruption of a tumour suppressor gene, the cell is considered to have undergone initiation. Expansion of this clone may result in a benign neoplasm, and further genetic changes can lead to a malignant neoplastic lesion(62). In human colorectal cancer mutation in several genes is required to subvert a healthy cell into a neoplastic lesion. Common genes involved include APC (adenomatoas polyposis of the colon) tumour suppressor gene, Kras and SMAD4 genes. Kras is a GTPase enzyme involved in cell signaling, growth and differentiation. SMAD4 is the transcription factor that controls cell signaling and the tumour protein (TP)53 which is involved in tumour suppression and cell cycle control(63).

Phytochemical consumption and gastrointestinal tract cancers: Epidemiological evidence

Gastrointestinal cancers are influenced by various lifestyle choices. Survey data support the commonly held belief that consumption of a balanced diet high in

fruits and vegetables and low in red meat is protective against a variety of cancers.

Esophageal cancer

In the recent WCRF report (2007) investigated the effect of non starchy vegetable intake on esophageal cancer risk. 5 cohort studies, 37 case control studies and 6 ecological studies were investigated. Of the 5 cohort studies three studies reported a decreased risk when the highest intake group was compared to the lowest intake level of vegetable intake. One of these cohort studies reported a significant reduction in risk of developing esophageal cancer (OR= 0.66 95% CI 0.44-0.99). The remaining 2 cohort studies reported a non-significant increase in risk of developing esophageal cancer when the highest intake level of vegetables was compared to the lowest level. Of the case control studies, 29 reported a decreased risk estimate for esophageal cancer when the highest intake group was compared to the lowest intake of non starchy vegetables. Of these 29 studies, fourteen reported a significant reduction in risk. Five of the case control studies reported a significant increase in risk for esophageal cancer when the high intake level was compared to the lowest level of non starchy vegetable consumption. A meta-analysis conducted on 5 of the higher quality case control studies resulted in a summary effect estimate of 0.87 (95%CI 0.72-1.05) per 50g/day increments of non starchy vegetable intake (comparing highest level of intake to lowest intake)(2).

Stomach cancer

The WCRF report (2007) summarized the findings of 10 cohort and 45 case control studies investigating the effect of consumption of non starchy vegetables on stomach cancer. None of the 10 cohort studies reported a significant reduction in stomach cancer risk when the highest level of vegetable intake was compared to the lowest level of vegetable consumption. A meta-analysis was conducted on 7 higher quality cohort studies resulting in a summary effect estimate of 0.98 (95% CI 0.91-1.06) when the highest vegetable intake was compared to the lowest level of intake. The report also examined 45 case control studies. Twenty-eight studies reported a statistically significant decrease in risk of stomach cancer when the highest intake level was compared to the lowest intake level. The remaining seventeen studies reported a non significant decrease in risk. Four studies reported a non significant increase in risk and one study reported no association. A meta-analysis was conducted on 20 case control studies resulting in a summary effect estimate of 0.70 (95% CI 0.62-0.79). This result indicates a reduced risk of stomach cancer with a high intake level of vegetables. The results of all case control studies indicate a dose response relationship between increasing vegetable intake and reducing stomach cancer risk (2).

Colorectal cancer

Seventeen cohort studies examined the effect of non starchy vegetable consumption on the risk of developing colorectal cancer. Of these studies,

eleven reported a reduced risk of colorectal cancer when the highest intake level was compared to the lowest intake level of non starch vegetables. Three studies reported a significant reduction in colorectal cancer risk when high intake was compared to low intake of vegetables. A meta-analysis was conducted on 6 studies, resulting in a summary effect estimate of 1.00 (95% CI 0.9-1.11) when high intake level was compared to lowest intake level (2).

In contrast to the above data, consumption of fruits and vegetables is inversely correlated with risk of developing colorectal cancer (64). This result is supported by meta-analysis showing that fruit and vegetable consumption is protective against gastric adenoma (65). Lunet (2005) also showed that although fruit and vegetable consumption is protective against the onset of gastric cancer for greater than 10 years following the onset and treatment for gastric cancer there was no apparent protection against the inevitable onset and mortality caused by gastric cancer, suggesting a genetic component in this type of cancer. An additional meta-analysis showed that greater than 2.5 servings of vegetables/day had a protective effect against gastric cancer (HR=0.56; 95% CI 0.34-0.93) when compared to those individuals who consumed less than 1 serving/day(66). In addition to the quantity of vegetables consumed daily the type of vegetable appears to have an affect on the risk of gastric cancer. Root vegetables appear to more protective (HR=0.43; 95% CI 0.27-0.69) that leafy vegetables (HR=0.64; 95% CI 0.42-0.99) when individuals consuming ≥3 servings were compared to ≤0.5 servings/week (67). This protective effect not only exists for fruits and vegetables. It also exists for, herbs, spices (68), teas (69) and garlic (70).

Green tea catechin consumption and gastrointestinal tract cancers

Tea is one of the most consumed beverages in the world. Recently it has been noted that the polyphenols contained in green tea catechin may provide a protective effect against gastrointestinal tumours. Meta-analysis for tea consumption have found that when the highest level of green tea catechin consumption was compared to the lowest there was a significant reduction in stomach cancer risk RR of 0.73 (95% CI 0.64-0.83) (71). The same protective relationship for colorectal cancer can be seen when the highest level of tea intake is compared to the lowest intake level (RR=0.82; 95% CI 0.69-0.98) (72). However, for lower doses of green tea catechin (5 cups/day) compared to no green tea catechin intake there appears to be no protective effect on gastric cancer incidence (OR = 0.99; 95% CI 0.78-1.27) (73). There is a large body of evidence supporting the protective role of phytochemicals against a variety of cancers including cancers of the gastrointestinal tract. In recent years green tea catechin has been implicated in a number of cancer preventative theories. In addition, its ease of consumption and ubiquitous nature has made green tea catechin an ideal target for further research.

Brewed tea from the *Camellia sinenis* plant is one of the most popular beverages consumed in the world. Brewed tea has 3 main forms, including white, green and black tea, all of which come from the same species of plant. They differ in how the leaves are processed to create the final product. In animal models tea

has been shown to inhibit a variety of cancers including esophagus, stomach, small intestine and colon (74). The active components of green tea catechin in cancer prevention are generally thought to be the catechins, including epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG) (figure 4).

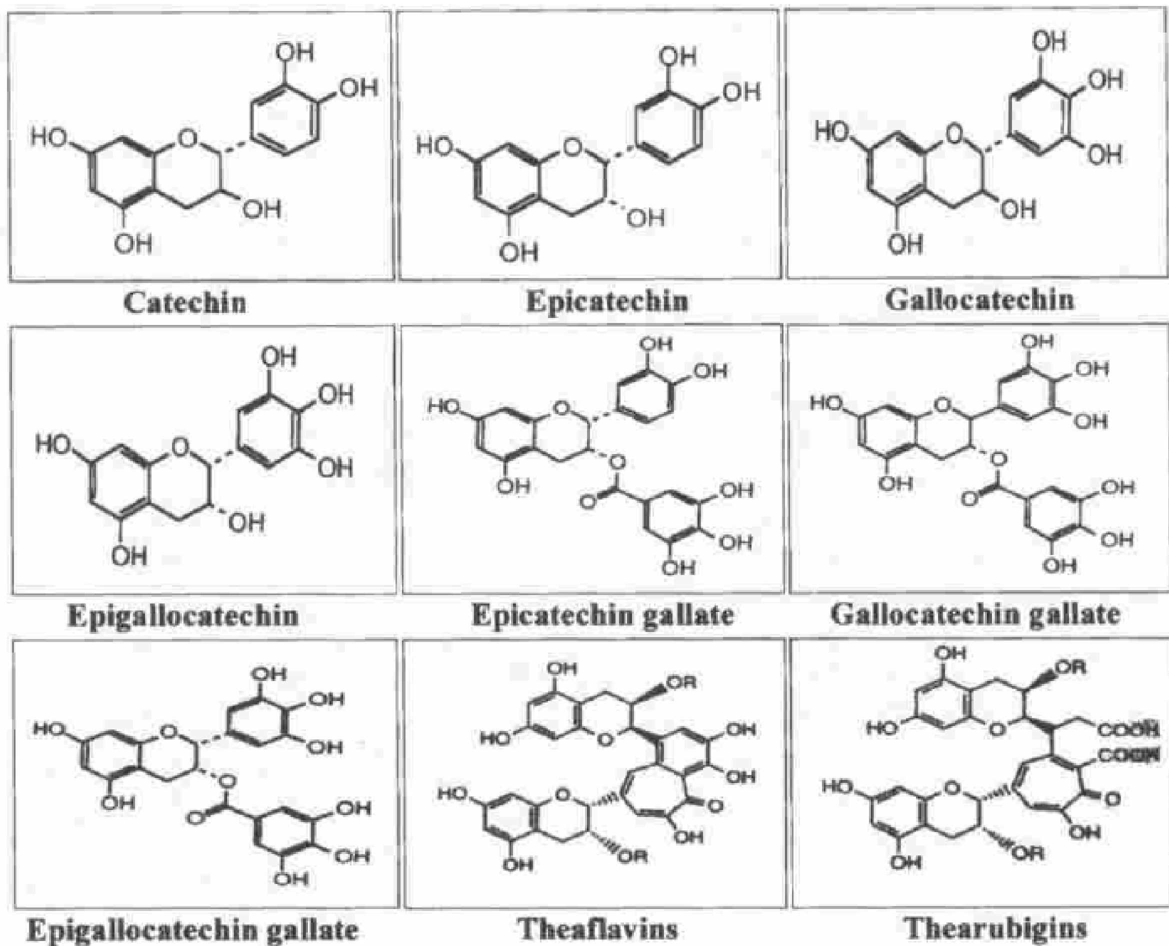

Figure 4: Catechin structures found in tea(75).

EGCG is present in the greatest quantity (76), amounting to 50-80% of the dry weight of catechins (47). The bioavailability of EGCG is approximately 3.5% and

the peak plasma level of EGCG is obtained 2 hours after consumption and the half-life is reported as between 3.5-5 hours. This poor bioavailability and short half-life is probably why continuous consumption of tea during the day, amounting to1.5 liters/d, is required to see optimal reduction of disease risk (77). In addition, the short half-life of EGCG is most likely the reason why repeated administration of EGCG is needed to alter xenobiotic metabolism (78). Most EGCG is recovered in urine as methylated, sulfated, and glucuronidated metabolites, following first pass metabolism in the liver and gastrointestinal tract tissues (77). In mice gavaged with [H^3] labeled green tea catechin and then sacrificed 24 hours later, measurable levels of [H^3] labeled EGCG were found in liver, kidney, lung and brain, showing that EGCG was incorporated into a wide variety of tissues (79).

EGCG mechanisms of action

There are several theories proposed for the mechanism of action for green tea catechin inhibition of cancer. Catechins have frequently been proposed to act directly as antioxidants in the quenching of reactive oxygen species or electrophilic metabolites of xenobiotics. Although catechins display antioxidant properties in vitro(80), it is unlikely that they can compete functionally with milli-molar concentrations of GSH in enzyme catalyzed reactions such as those catalyzed by glutathione peroxidase or GST isozymes. Catechins could decrease cellular oxidant stress by causing low levels of stress and thereby inducing oxidant defense enzyme systems. EGCG is proposed to competitively

inhibit the phosphorylation of mitogen activated protein kinases (MAP2K1, ERK1, ELK1) and c-Jun and this may in turn influence cell division, differentiation and apoptosis. In addition, in vitro models have proposed that EGCG blocks the function of epidermal growth factor receptor (EGFR). The EGFR plays a role in cell growth and differentiation and it is often over expressed neoplastic cells. Over expression of EGFR is proposed to be a contributing factor in the uncontrolled division of some neoplastic cells (74). During cancer development the normal cell cycle often becomes disregulated, with shortened quiescent periods and prolonged growth periods. EGCG is thought to trigger the onset of the quiescent periods G_0/G_1 in a variety of tumour cell lines including breast, epidermoid, and prostate cells by inducing the expression of p21 and p27, which inhibit the cyclin dependant kinases responsible for cell cycle regulation (74;79). EGCG is also proposed to play a role in inhibiting proteosomes and matrix metalloproteases (MMP). Proteosomes are involved in degrading cell signaling molecules such as IkB (inactivates NFkB) and cyclin D1 (cell cycle regulator). MMPs are involved in tumour progression and metastasis. MMPs and proteosomes are often up regulated in aggressive cancers. Inhibiting both proteosomes and MMPs could prevent tumour expansion (74).

EGCG is also proposed to play a role in cell apoptosis. Nuclear factor κB (NFκB) is responsible for regulating the body's immune response to infection and it plays a role in suppressing apoptosis in neoplastic cells. In tumour cell lines EGCG has been shown to inhibit the constitutive activation of NFkB thus allowing apoptosis of these cells (74). A healthy biological system exists in a balance of

cell survival and apoptosis. In neoplastic cells the balance is tipped towards survival of cells that are generally not healthy. EGCG may enhance the activity of an apoptotic signaling protein called caspase 3 although the exact mechanism is still unknown (74). EGCG is also proposed to inhibit neoplastic cell invasion by inhibiting the process of epithelial to mesenchymal transition (EMT). EMT is the first stage in promoting a noninvasive neoplasm to an invasive neoplasm. EGCG may block the role of AHR in activating the transcription factor Slug, which in turn suppresses the transcription of E-cadherin. E-cadherin is a tumour suppressor glycoprotein involved in cell-cell adhesion. Loss of E-cadherin function or suppression of production is required for the onset of EMT (81)

The effect of EGCG on xenobiotic metabolism

An additional mechanism of cancer prevention is the inhibition of cancers derived from exposure to common secondary carcinogens like PAH, HCA and NA. EGCG may accomplish this in part by interacting with the aryl hydrocarbon receptor (AHR). As mentioned previously, HCA and PAH bind to the AHr to promote transcription of the CYP1A1 and CYP1A2 genes. Recent research has investigated the relationship between Hsp90 and EGCG. EGCG is thought to inhibit the dimerization of AHR with the aryl hydrocarbon receptor nuclear translocator thus inhibiting the upregulation of CYP1A1 and 1A2 and 2E1 (48;82). Inhibiting CYP1A1, 1A2 and 2E1 will effectively prevent the bioactivation of PAH, HCA and NA which will in turn prevent adduct formation and tumour promotion. In an experiment using HepG2 cells EGCG inhibited the transcription

of CYP1A1 promoter driven reporter gene. CYP1A1 expression was induced through the known AHR ligand 2,3,7,8, tetrachlorodobenzo-p-dioxin (TCDD). As a result EGCG prevented the accumulation of CYP1A1 and 1A2 mRNA that would normally have occurred following the binding of AHr to DNA. It was postulated by the authors of this experiment that EGCG prevents AHr from binding to DNA and inducing the expression of CYP1A1 and 1A2. Consequently it is believed that EGCG is preventing the bioactivation of PAH, HCA and NA by inhibiting the expression of CYP1A1, and CYP1A2 genes (83). In a recent in vitro experiment, EGCG was also shown to decrease the transcription of the AHR gene. AHR expression requires the co-expression of CK2 and c-Rel. EGCG can inhibit the activation c-Rel and CK2 thus lowering or preventing the expression of AHR (81). This results in the less AHR being present in the cytosol for PAH to activate, which in turn results in lower expression of CYP1A1/2, and decreased bioactivation of PAH.

EGCG also interacts with NRF2. Addition of EGCG to colon cancer cells in vitro results in an upregulation of UGT. UGT was also up regulated in BALB/c mice gavaged with EGCG (36). The exact mechanism by which EGCG causes this increase is not known, but it is widely accepted that EGCG facilitates the phosphorylation of the serine and threonine residues of NRF2 thereby causing its translocation to the nucleus where it binds to ARE thus resulting in UGT being upregulated (52). Upregulation of UGT would result in an increased ability to conjugate any nucleophilic metabolites of PAH and HCA, thus facilitating their elimination.

EGCG is proposed to provide protection against HCA, PAH and NA induced carcinogenesis by inhibiting CYP mediated bioactivation and by upregulating phase 2 conjugation enzymes, thereby reducing the amount of reactive intermediates present in the cell. Reducing the amount of time that reactive intermediates are in the cell reduces the risk of DNA adduct formation which in turn reduces the potential for cell transformation into a neoplastic cell.

Summary and Rationale

There is sufficient epidemiological evidence to suggest that consumption of cooked and cured meats is associated with an increased risk of developing gastrointestinal tract cancers. It is also accepted that consumption of phytochemicals in green tea catechin is associated with a reduced risk of gastrointestinal tract cancer. However, the epidemiology in both of these areas remains somewhat controversial and, by its nature, does not prove that the relationships are causative. The interactions between these families of chemicals is still poorly defined, and there are no established whole animal models for induction of cancer by cooked/cured meat consumption. Bioactivation can occur in any cell that contains the appropriate CYP enzymes, in this case CYP1A1, 1A2 or 2E1. Once ingested, secondary carcinogens such as BaP, PhIP and DMN pass through the esophagus, stomach, small intestine and large intestine; bioactivation can occur in any of these tissues resulting in genotoxic stress in that tissue. Chronic exposure to complex mixtures of secondary

carcinogens and phytochemicals can significantly alter gene expression and change phase 1 and 2 xenobiotic metabolism.

In the following experiments we set out to first establish that feeding cooked and cured meat to rats does in fact result in gastrointestinal tract cancers. In addition, we created a functional food that would combine the protective effects of green tea catechin into a potentially carcinogenic cooked and cured meat. We hypothesized that the addition of phytochemicals from green tea catechin into hot dogs may negate the potential carcinogenicity of the meat item. These two hypotheses were tested in a long term morbidity trial. We further hypothesized that our green tea catechins would reduce CYP bioactivation and enhance conjugating enzymes, and these hypotheses were tested in short term feeding trials.

Chapter 2: The effect of lifetime co-consumption of cooked/cured meat and green tea catechins on the incidence of gastrointestinal cancers in male F344 rats

Abstract

Lifetime consumption of cooked and cured meat has been associated with increased risk of gastrointestinal cancer in human populations. Consumption of green tea catechins, like EGCG, has been associated with prevention of gastrointestinal cancers. One hundred and eight male F344 rats were weight matched and placed on diets designated as control (CON), control containing green tea catechins (CGT), hot dog (with nutrient supplements) (HD) or hot dog containing green tea catechins (HDGT). Hot dog was fed at the level of 50% of dry matter intake. Rats were fed these diets from 21 days of age until predetermined morbidity endpoints were met, where upon they were euthanized and all organs and tissues excluding skeletal muscle were fixed, sectioned and stained. The slides were examined for evidence of cancer development. Fisher's exact test and Kaplan-Meier survival analysis was used to determine the relationship between diet and cancer. In conclusion, rats fed HD and HDGT diets had a lower incidence of large granular lymphocytic (LGL) leukemia than rats fed the control diet. In addition, rats fed HD and HDGT diets did not have gastrointestinal cancers.

Introduction

Cancers of the gastrointestinal tract (esophagus, stomach, small and large intestine) comprise approximately 17% of all cancer-related deaths(1;84). There is strong evidence that cancers related to the gastrointestinal tract are associated with lifestyle factors such as low vegetable consumption and the consumption of cooked, processed or cured red meats.

Recent meta-analysis examined the risk of developing gastrointestinal cancer associated with meat consumption. The relative risk for gastrointestinal cancer ranged from 1.21 (95% confidence interval (CI) of 1.13-1.29) to 1.43 (95% CI of 1.04-1.94). In addition, the estimated risk for increasing meat consumption by 100g/day was 1.29 (95% CI 1.04-1.60) for colorectal cancer (2;3). For other gastrointestinal cancer sites the evidence is less compelling. World Cancer Research Fund (WCRF) meta-analysis indicates that there is limited evidence that associating the risk of developing esophageal cancer with consumption of red meat(2). The same report indicates that the main association of stomach cancer and meat comes with the consumption of processed meat and not red meat consumption(2).

Red meat is generally consumed cooked and/or cured/ processed. Cooking of meat reduces potential pathogen exposure and enhances flavor. However, epidemiological evidence indicates that cooking can increase the carcinogenic potential of meat(6;7;9-11;85;86). The main cause of this increased risk is

associated with the heterocyclic amines (HCA), polycyclic aromatic hydrocarbons (PAH) and nitrosamines (NA) contained in cooked and cured red meat.

HCA, PAH and NA are lipophyllic secondary carcinogens that are present in cooked and preserved meats such as sausages, hot dogs and smoked cold cut meats (19). Once ingested these molecules are metabolized to their carcinogenic forms by a process called bioactivation or biotransformation. Dietary xenobiotics must pass through the gastrointestinal epithelium before entering the hepatic portal circulation or being released into the lymphatic flow as a chylomicron. Both of these routes will bring the xenobiotic to the liver, where the capacity for phase I and II metabolism is dramatically higher than that of the gastrointestinal tract and other tissues. To the extent that the gastrointestinal tract does express P450 enzymes, some portion of dietary secondary carcinogens may be bioactivated before reaching the liver. This makes gastrointestinal tract tissues potential targets for cancers induced by dietary sources of HCA, PAH and NA. PAH and HCA are metabolized by CYP1A1/A2 and then conjugated to glutathione by glutathione S-transferase (GST) to allow for excretion via the kidneys. NA is metabolized by CYP2E1 and then conjugated to UDP glucuronide by UDP-glucuronyl transferase (UGT)(41).

The green tea catechin EGCG is thought to play an important role in inhibiting the upregulation of CYP1A1/A2 and CYP2E1. This potential inhibition could prevent transcription and translation of the appropriate CYP genes thus reducing the amount of PAH, HCA and NA biotransformation in the cell. EGCG is also demonstrated to upregulate GST and UGT(48;82) which would result in an

increase in conjugation and excretion of xenobiotic metabolites. The combined effect of inhibition of biotransformation and enhanced excretion may reduce the carcinogen burden faced by the animal.

Our objective for the following experiment was to determine whether the co-consumption of cooked/cured meat and green tea catechins prevents the formation of gastrointestinal cancer in male, F344 rats.

Methods

Animals

One hundred and eight male weanling F344 rats were purchased from Charles River, Canada. Animals were housed in environmentally enriched cages in accordance with the Animal Care Committee at the University of Guelph, Ontario, Canada. Wire bottom cages (41.25x26.25x14.5 cm) were supplemented with environmental enrichment consisting of nesting boxes, newsprint paper, toys and wooden blocks. Animals were weight matched and assigned to one of four treatment groups. See table 5 for an outline of animal numbers allocated to treatment groups. Health of the animals was monitored by weighing three times a week, palpation and daily health assessment (Appendix 1).

Diets

The 4 diets consisted of control (CON), control containing 0.5% green tea catechins (CGT), hot dog (HD), and hot dog containing 1% green tea catechins (HDGT) (tables 1-6). The HD diet consisted of a 1:1 ratio of grilled pork hot dog

containing 10% fat and a supplement containing vitamins, minerals and essential fatty acids added to meet the levels outlined in AIN93g (87). Tables used to balance nutrients can be found in appendix 3. The HDGT diet consisted of a 1:1 ratio of grilled 10% fat pork hot dog containing 1% w/w green tea catechins and the same supplement of vitamins, minerals and essential fatty acids for a total dietary concentration of 0.5% green tea catechins. The CON diet was designed to provide the same levels of nutrients found in the HD diet, using casein, free amino acids and lard to replace the protein and fat of the hot dog component. The CGT diet was similar to the CON diet, with the addition of 0.5% w/w green tea catechins (Sunphenon 90LB; Taiyo Green power, Jiangsu, China; certificate of analysis in appendix 5), This level of catechins was designed to mimic the optimal dose of green tea catechin in humans (estimated to be 10 cups/day or 1.5 litres) (69). This calculation used to convert human exposure to rat feeding is shown in appendix 2. All diets were mathematically balanced in the major nutrient classes and designed to be isocaloric. See appendix 3 for details on diet balancing and analysis.

Pork hot dogs were made by the Herman Laue Spice Company (Uxbridge, Ontario, Canada). The hot dogs contained 10% fat, used corn starch as a binder and contained 150 ppm sodium nitrite. Both types of hot dogs were smoked in a commercial smoker for 30 min at 155ºC. Hot dogs containing green tea catechins had 1% w/w green tea catechins added to the hot dog mixture during the blending stage just prior to injecting the mixture into the casing and prior to the smoking stage. After smoking the hot dogs were packaged and frozen until

use. Prior to feeding, both hot dogs and hot dogs containing catechins were grilled on standard counter top grills (Hamilton Beech) for 30 min at 350ºC. Grilled hot dogs were then frozen at -20ºC until needed. Hot dogs were thawed in the fridge prior to feeding. Animals were fed at a certain percentage of body weight in order to maintain even intake and growth among individuals. This percentage was adjusted downward over time to approximately 95% of *ad libitum* intake, so that all of the rats were consuming their allotment.

Table 1: Diet composition for control (CON) and control green tea catechin diet (CGT). CON diet is based on AINg93 which has been balanced to match the nutrient content of the 10% fat pork hotdog + supplement containing vitamins, minerals and essential fatty acids. CGT diet is the control diet containing 0.5% green tea catechins. Diets were fed to control animals until predetermined cancer endpoints were met.

Ingredients	Control Diet (CON) % inclusion	Control + 0.5% green tea catechins (CGT) % inclusion
Cerelose	18.8	18.3
Casein	44.5	44.5
Celufil	5	5
Vitamin Mix	3	3
Mineral Mix	10	10
L-Cysteine	0.3	0.3
L_Histidine	0.5	0.5
L-Arginine	1	1
L-Lysine	0.7	0.7
Safflower oil	3.4	3.4
Flax oil	0.8	0.8
Lard	12	12
Green tea catechins	0.0	0.5
Total	100	100

Table 2: Diet composition for Hot dog (HD) and Hot dog containing 1% green tea catechins (HDGT). Hot dog diets were based on AINg93 which has been balanced to match the nutrient content of the 10% fat pork hotdog + supplement containing vitamins, minerals and essential fatty acids. Diets were fed to rats until predetermined cancer endpoints were met.

Ingredients	Hot dog diet (HD) % inclusion	Hot dog diet +1% green tea catechins (HDGT) % inclusion
Cerelose	32.3	32.3
Celufil	5	5
Vitamin Mix	1.5	1.5
Mineral Mix	5	5
L-Proline	2	2
Safflower oil	3.4	3.4
Flax oil	0.8	0.8
Grilled hot dog (Dry matter basis)	50	0
Grilled hot dog (1% green tea catechin) (Dry matter basis)	0	50
Total	100	100

Table 3: Vitamin premix added to all diets. The premix was added at 3% w/w to the control diets and at 1.5% w/w to the powdered component of the hot dog diets.

Ingredients	Control Diet g/100g	Hot dog diet g/100g
Thiamin	0.0509	0.0000
Riboflavin	0.0214	0.0100
Nicotinic acid	0.2990	0.0000
Ca pantothenate	0.0530	0.0100
Pyridoxine	0.0270	0.0000
Folic acid	0.0120	0.0200
Vitamin B-12	0.0001	0.0001
Retinol	0.0004	0.0080
Vitamin D	0.0001	0.0001
Vitamin E (α-tocopherol)	0.1790	0.3580
Vitamin K (phylloquinone)	0.0030	0.0060
Choline	3.5600	7.1300
Biotin	0.0007	0.0010
Sucrose	95.7770	92.4000
Total	100.0000	100.0000

Table 4: Mineral premix added to all diets. Control diets contained 10% w/w mineral mix and hot dog diets contain 5% w/w in the powdered portion of the diet.

Ingredients	Control diet g/100g	Hot dog diet g/100g
Calcium carbonate	13.360000	26.29
Ferric citrate	0.290000	0.172
Magnesium oxide	0.910000	0.782
potassium phosphate monobasic	16.220000	0.000
potassium citrate, tripotassium monohydrate	7.880000	0.000
Sodium chloride	5.850000	0.000
Zinc carbonate	0.070000	0.052
Cupric carbonate	0.010000	0.026
Manganous carbonate	0.020000	0.052
Sodium selenate anhydrous	0.001000	0.000
Potassium sulfate	1.740000	3.49
Potassium iodate	0.000300	0.0007
Ammonium paramolybdate 4H20	0.000200	0.00059
Sodium meta-silicate 9H20	0.054000	0.108
chromium potassium sulfate 12H20	0.010200	0.02
Sodium fluoride	0.002000	0.004
Nickel carbonate	0.001000	0.002
Boric acid	0.003000	0.006
Lithium chloride	0.000065	0.001
Ammonium vanadate	0.000200	0.0004
Sucrose	53.510000	68.98
TOTAL	100.000000	100

Cancer endpoints

Rats were fed controlled amounts of one of four diets CON, CGT, HD or HDGT diets until they displayed termination end points. Cancer endpoints were predetermined under the guidelines set out by the Canadian Council on Animal Care. Rats were to be euthanized when tumour size >10mm diameter, or morbidity end points such as diarrhea, lethargy, pain or guarding, or 5% peak body weight loss were displayed. Once endpoints were met animals were then euthanized and necropsies were performed on all animals. At the time of death all tissues were examined under 10x magnification and any tissues that had an abnormal morphology or obvious tumour were retained for examination. In addition to suspect tissues, samples of brain, esophagus, stomach, small intestine, large intestine, kidneys, liver, spleen and testes were retained and fixed in 10% phosphate buffered formalin (Fisher Scientific, Ottawa, On, Canada). All tissues were examined for the presence and type and stage of cancer.

Tissue handling

Stomach, small intestine, and large intestine were emptied of contents and flushed with ice cold phosphate buffered saline and then flushed with 10% phosphate buffered formalin. Tissues were then stored at room temperature in 10% phosphate buffered formalin until needed. All tissues were trimmed and placed in cassettes and then sent to the Animal Health Laboratory, University of Guelph, ON, Canada to be prepared for paraffin embedding and sectioning and

histology. Slides were stained with hematoxylin and eosin. Histological samples were then assessed for type, stage and grade of cancer by a rodent cancer specialist, Dr. Geoff Wood, Pathobiology, Ontario Veterinary College, University of Guelph, ON, Canada.

Statistical Analysis

Given the high daily intake of grilled, nitrite preserved meat, we thought that cancer morbidity would be common by approximately 18 months of age. This was basically speculative given that no one has, to this point, published data on practical feeding trials of this nature. Beyond 18 months of age we started to see animals exhibiting typical old age endpoints of muscle wasting, arthritis and loss of appetite. Consequently some of the animals that were euthanized because of weight loss were actually suffering from the various effects of old age other than cancer. As a result the method chosen for the statistical analysis of this study allows us to censor non-cancer data points to focus on selected disease processes. It is only after sacrifice and tissue harvesting that we can determine that there was no cancer present in any of the organs harvested, but the termination criteria are a critical aspect of proper animal care for this type of experiment.

Growth data was normally distributed according to the Levene statistic and the results were analysed using an analysis of variance with a Tukey HSD (honesty significant difference) post hoc test. Cancer incidence data were analysed using Fisher’s exact test. Survival data were analyzed using Kaplan Meier analysis

and Generalized Wilcoxon test. All data were analyzed using SPSS 17.0 statistical software package. A $p \leq 0.05$ was considered significantly different and $p \leq$ value less than 0.10 is considered a trend.

Kaplan Meier survival analysis

Kaplan Meier analysis is a statistical assessment of time until a certain event. It is often used in cancer studies because it characterizes how long it takes until specific end points are reached. Often in survival studies mortality points will be removed from a study because the individual reached an endpoint that was not being assessed. For example, if an animal was sacrificed but had no cancer endpoints, then its data would be considered cancer-free up to that point, and the animal would be subsequently "censored" or removed from the study without contributing to mortality. Given that the necropsy process was very thorough, and we were convinced that no significant neoplastic conditions were present in those rats at the time of removal from the study, their data are reasonable to include up to that point as being cancer-free. One has to be cautious with the description of censored data and aware of the potential for post-hoc interpretations, but censoring is generally required for long-term studies with multiple causes of morbidity or mortality.

Censoring

In this study we found that some animals were developing large granular lymphocytic (LGL) leukemia while most of the other rats did not develop any

cancers. LGL leukemia is a cancer that F344 rats develop as they age (Ward et al, 1982). In some cohorts, approximately, 80% of F344 rats will develop LGL leukemia as they age (88). As a result, we examined whether there was a relationship between diet and LGL leukemia incidence. Animals that did not have any LGL leukemia were censored allowing us to determine whether diet was affecting the incidence of LGL leukemia.

Combining controls

In this study we chose to have small number of animals in the two control groups so we could maximize the number of rats used in the treatment groups that we expected to have more significant mortality rates. Using a low number of control animals is often done in mortality/morbidity studies when it is not expected that the control group will exhibit significant mortality during the experimental period. In the case of feeding hot dogs to rats there was a reasonable expectation, given the levels of nitrite/nitrosamine, PAH and HCA that rats were consuming, that the HD and HDGT animals would reach cancer endpoints before the animals consuming the control diet. However, this was not the case as animals on the experimental diets lived longer than the animals on the control diet. To help correct for this imbalance in animal numbers we examined whether we could combine our control groups to generate more statistical power.

Results

Additional endpoints

Animal health assessment sheets (appendix 1) were maintained for all animals. Any abnormal behavior or symptoms were noted prior to euthanasia. Many of the animals in our study did not show any signs of ill health prior to euthanasia other than the 5% body weight loss. Weight of the animals was monitored 3 times a week and percent weight loss was calculated at that time for each animal. When calculating % body weight loss in the experimental animals only those with sustained and escalating body weight loss were euthanized as weight loss endpoints. During the course of the 900 day experiment rats would often gain and lose between 3-5% (10-15g) of their body weight. This fluctuation in body weight was attributed to variation in weighing time (in relation to feeding and drinking) and minor reversible health issues. If rats showed signs of regaining their body weight within 48 hours of their previous weight loss, or they did not continue to lose weight, they were not euthanized as a weight loss end point.

The study lasted a total of 900 days. At day 352 one control green tea catechin rat was euthanized because of a hind limb injury. Samples were not retained for necropsy. Over the course of the experiment 2 rats died (one HD and one HDGT) suddenly over night. Both rats had leukemia but the degree of tissue decomposition made it difficult to determine whether they had any secondary cancers.

Rats were fed diets starting at 21 days of age until one of the defined morbidity endpoints was displayed (between 300 and 900 days of age). The rats grew well and attained a normal adult weight (Figure 5). There were significant differences in mean weight between treatment groups over the course of the experiment. The average weight of the CON animals was less than HD ($p = 0.002$) or HDGT ($p>0.0001$) rats. The mean weight of CON rats was not different from CGT rats.

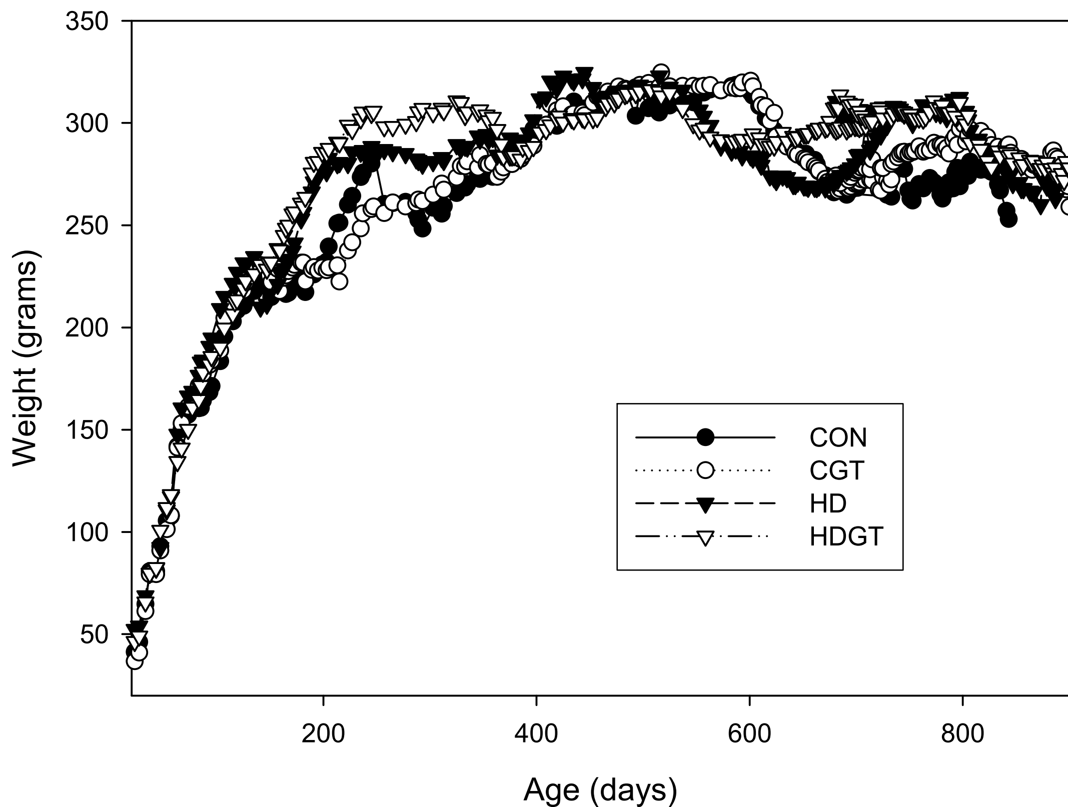

Figure 5: Increase in rat weight over time as an indicator of growth and general health. Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); a HD (hot dog diet fed 1:1 with supplemented nutrients); or HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age until morbidity endpoints were reached.

When a morbidity endpoint was met histopathology was used to determine the presence and type of cancer. A general overview of the occurrence and type of cancers seen in the rats is shown in Table 5. In general, rats had a large number of LGL leukemias.

Table 5: Summary of cancer type and frequency. Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); a HD (hot dog diet fed 1:1 with supplemented nutrients); or HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed from 21 days of age until morbidity endpoints were reached.

Dietary Groups	CON	CGT^{AF}	HD^{BDGI}	HDGT^{CEHJ}
Total # of rats (n/treatment)	10	16	41	41
Large granular lymphocytic leukemia	6	6	14	3
Adenoma	0	1	2 (Cecum& Stomach)	0
Fibrosarcoma	0	1	1	0
Soft tissue sarcoma	1	0	0	0
Liver	0	1	0	0
Testicle	0	1	0	0
Pancreas	0	0	1	0
Osteosarcoma	0	0	1	0
Pre-neoplastic lesion	0	2 (Liver)	2 (Liver)	1(Stomach)
Benign lesion	0	0	0	1 (Skin)
Non cancer-related death	3	4	21	37
Total Malignancies	7	12	20	4

Total Leukemias (as % of Malignancies)	86	50	70	75
Leukemia incidence (as % of rats)	50	37.5	34.1	7.32

Fisher’s exact test 2 tailed significance comparing total malignancies of:

A: CON and CGT $p=0.644$

B: CON and HD $p=0.300$

C: CGT and HDGT $p<0.0001$

D: Ccon and HD $p=0.04$

E: Ccon and HDGT $p<0.0001$

Fisher’s exact test 2 tailed significance comparing leukemias of:

F: CON and CGT $p=0.422$

G: CON and HD $p=0.163$

H: CGT and HDGT $p=0.011$

I: Ccon and HD $p=0.441$

J: Ccon and HDGT $p=0.001$

All Cause Morbidity

The experiment ended when the oldest rat was 900 days old. Survival analysis was conducted on all rat data to determine whether feeding the diet altered the amount of time that each treatment group lived.

In figure 6 we see an overall comparison of all treatment groups for all cause morbidity. A Generalized Wilcoxon post hoc test indicated that there was not a statistically significant difference ($p=0.058$) in survival time between the treatment groups, although the p value could be considered a trend.

Kaplan Meier and Generalized Wilcoxon tests do not allow for pair-wise comparisons between diets when more than 2 treatment groups are analysed. One approach to improving the statistical power of the model is to combine our control groups, which can be considered if they do not differ statistically from each other. This will allow us to have a larger control group, which became limiting in our design due to unexpected survival in the HD and HDGT groups.

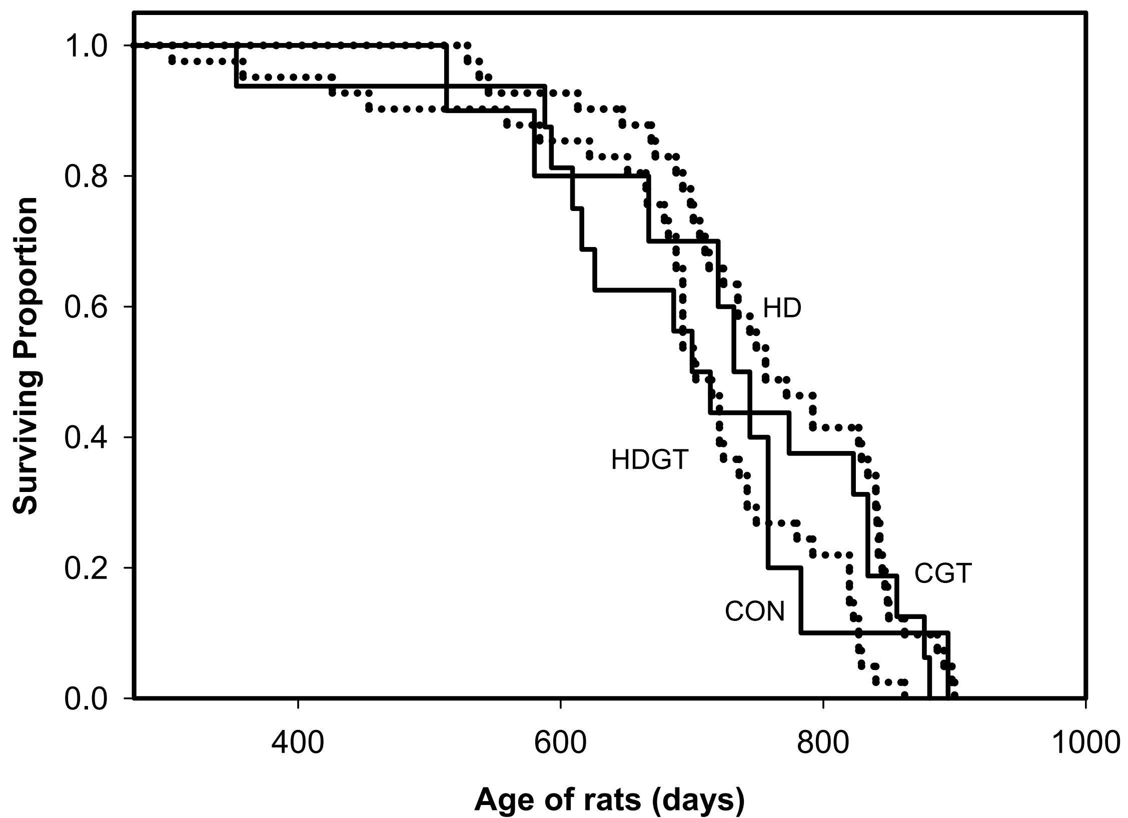

Figure 6: The effect of consumption of CON, CGT, HD or HDGT diets on the survival time of F344 rats (p=0.058). Diets: CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins); HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT(hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined morbidity endpoints were reached.

Before we continue with the analysis of our control groups, HD and HDGT we must first confirm that there is no statistical difference between CON and CGT (figure 7). When we tested CON and CGT using Kaplan-Meier analysis and Generalized Wilcoxon post hoc test we found that there was no significant difference in survival time between these 2 groups ($p=0.958$). This result confirms our ability to consider the two control groups as one thus increasing statistical power. As a result the following comparisons are between HD and HDGT and a Combined control group (Ccon).

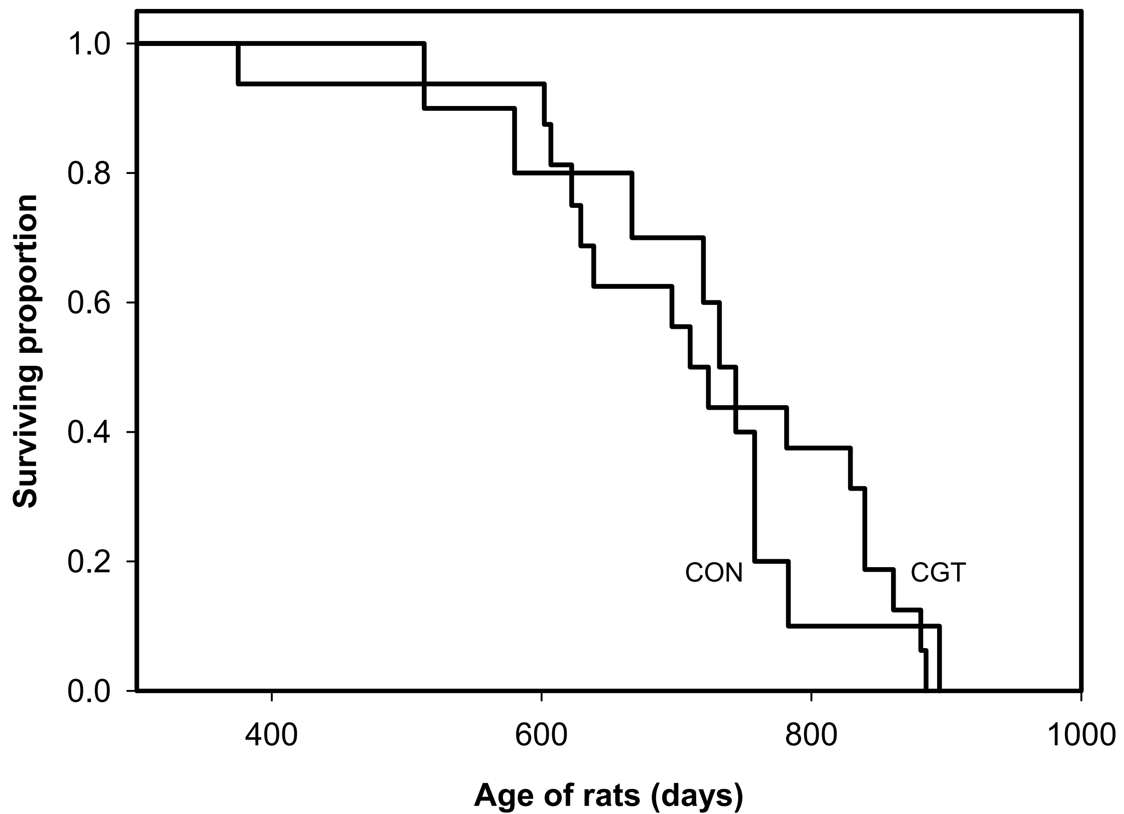

Figure 7: A pairwise comparison of survival time between CON to CGT p=0.958.Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

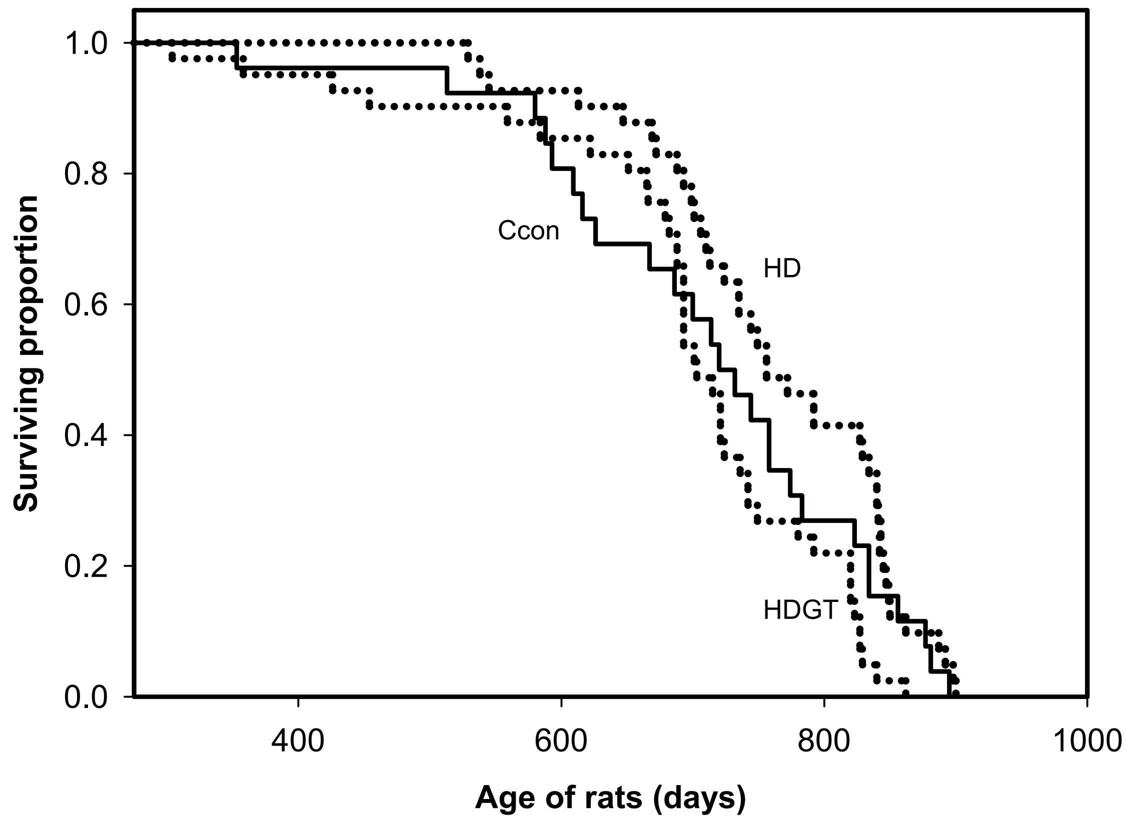

Figure 8: A comparison of survival time between Ccon, HD and HDGT (p=0.024). The survival times for CON and CGT were not statistically different and therefore their survival data has been combined into one group, Ccon. Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

In figure 8 we see a comparison between combined control groups to each of the hot dog diet groups. The Kaplan Meier analysis and Generalized Wilcoxon post hoc analysis indicates a significant difference between the group survival times (p=0.024). However separate pair-wise analysis is required to determine where the significance in survival time between Ccon, HD and HDGT is located. In addition, Kaplan-Meier and a Generalized Wilcoxon were designed to be used as a paired analysis and consequently the treatments should be paired to ensure an accurate reporting of results. Figure 9 illustrates the pairing of Ccon and HD.

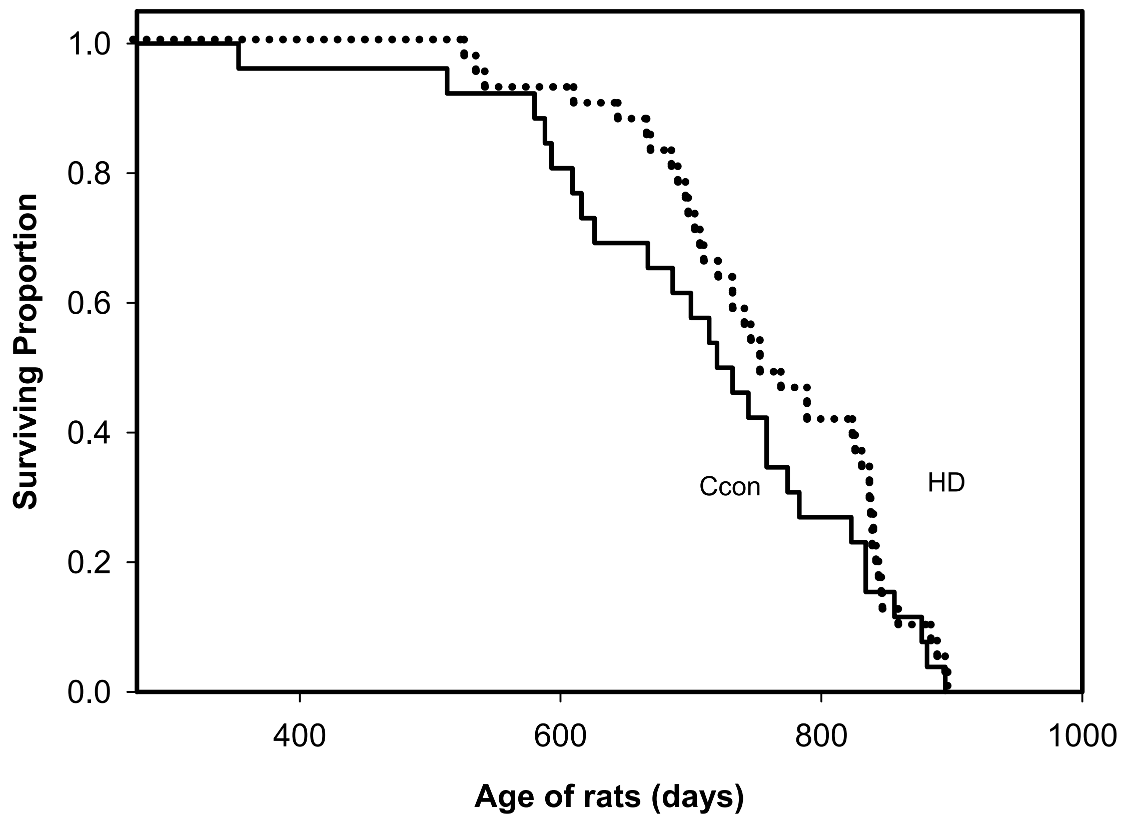

Figure 9: Shows a comparison in survival time between Ccon and HD (p=0.119). The survival times for CON and CGT were not statistically different and therefore their survival data has been combined into on group Ccon. Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

The survival time for HD was not significantly different from Ccon $p=0.119$.

Figure 10 shows a pair-wise comparison in survival time between Ccon and HDGT groups.

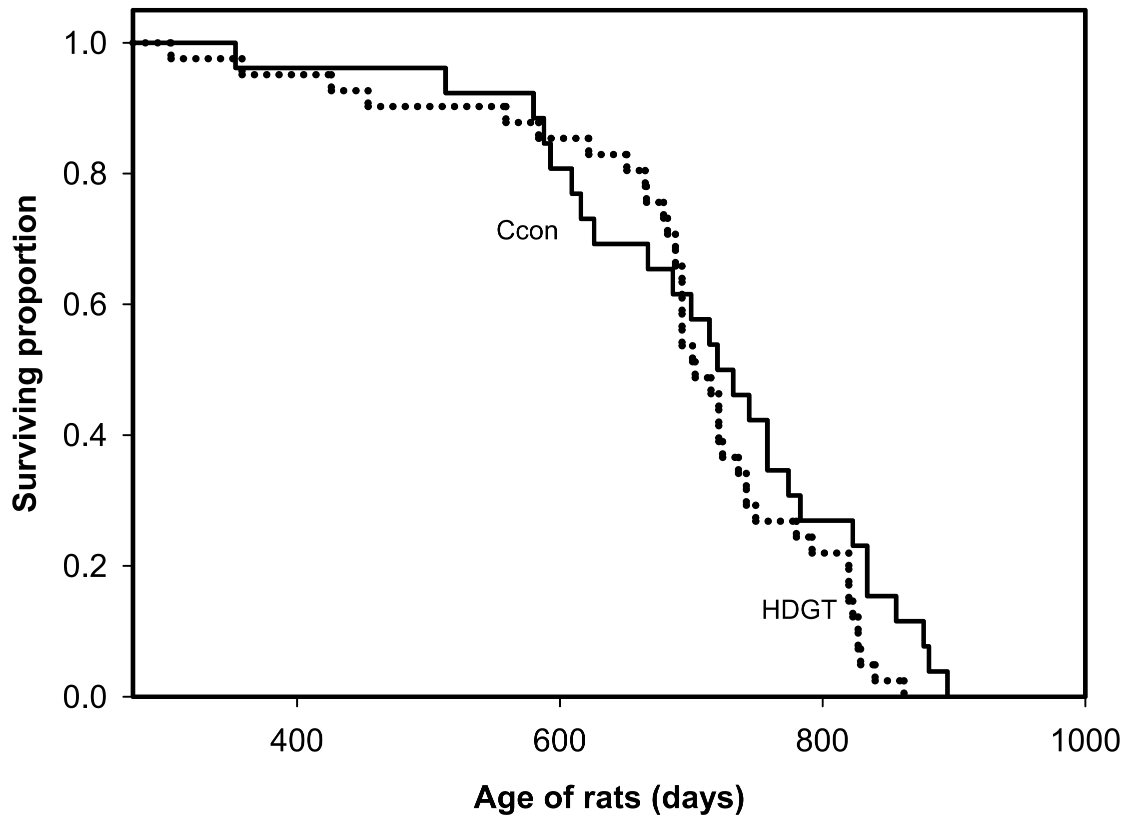

Figure 10: A comparison in survival time for all cause morbidity of Ccon and HDGT p= 0.567. The survival times for CON and CGT were not statistically different and therefore their survival data has been combined into on group Ccon. Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

The comparison of all cause morbidity survival time between Ccon and HDGT is not significantly different p=0.567 (figure 10).

Morbidity related to all cancers

The analysis of all cause morbidity does not allow for the examination of how dietary treatment may have been related to a particular cause of morbidity. Histopathology findings indicated that many of the rats died of endpoints not related to cancer. Thus we need to consider whether these non related causes of morbidity are affecting the statistical outcome of our analysis. Kaplan Meier statistical analysis allows us to examine a specific end point in a survival analysis through the method of censoring. Censoring a mortality endpoint leaves the survival data up to that point in the analysis, while not adding to the mortality. This is appropriate when an individual dies for a cause unrelated to the endpoint of focus, or in human studies, when an individual is lost to follow up.

In Figure 11 shows a comparison of all cancer-related survival times. All non-cancer-related endpoints have been censored. A censored data point is shown as an inverted triangle on the graph. Kaplan Meier and Generalized Wilcoxon post hoc analysis indicates a significant difference between survival times of rats with all types of cancer (p=0.0001).

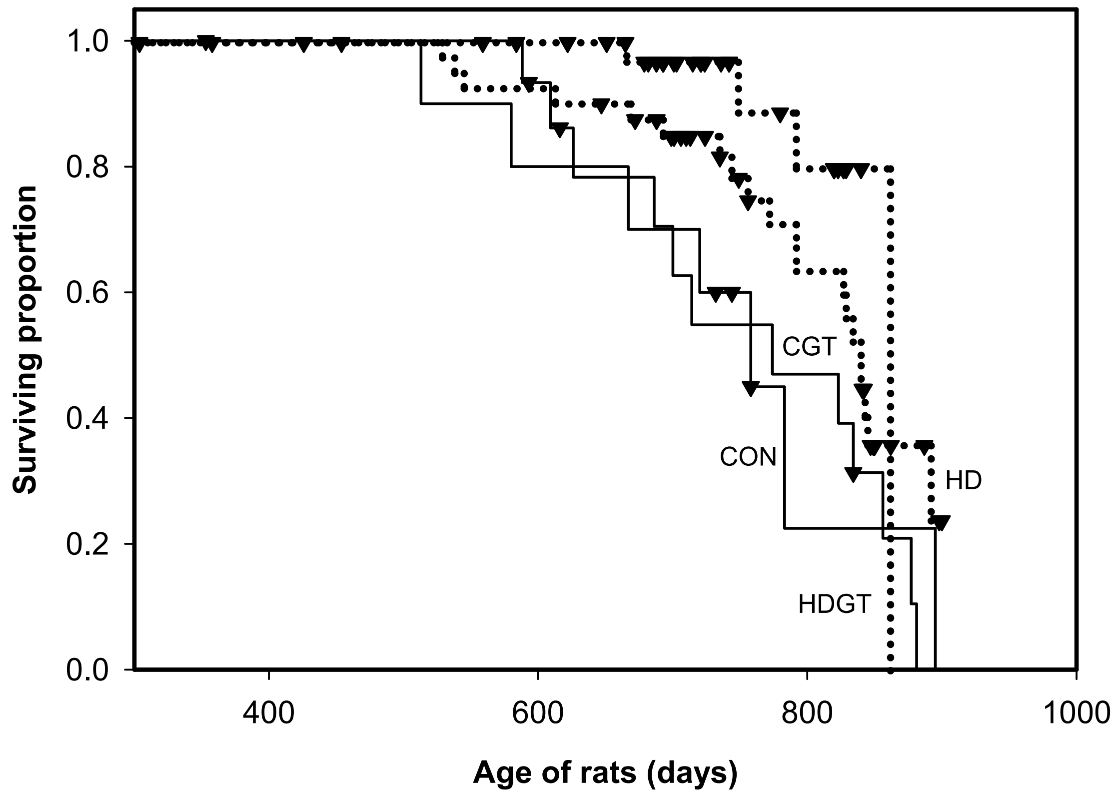

Figure 11: Comparisons of all cancer-related survival times for all treatment groups p>0.0001. All non cancer-related morbidity is shown as a censored data point illustrated by ▼. Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

As in our analysis of general morbidity we can combine our control groups to enhance the statistical power of our control group. A comparison between CON and CGT for all cancer-related morbidity was conducted and no significant difference in survival time was found (p=0.784) (figure 12).

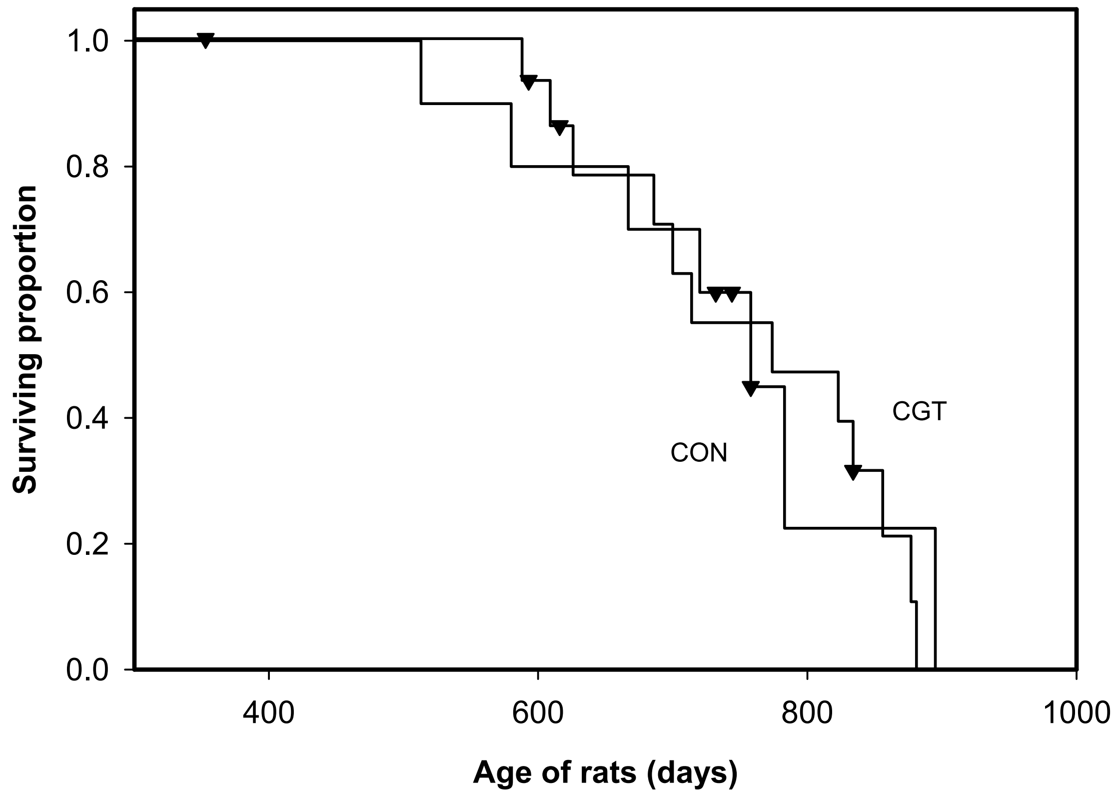

Figure 12: A comparison between survival time of CON and CGT for all cancer-related morbidity p=0.784. All non cancer-related morbidity is shown as a censored data point illustrated by ▼ p=0.799. Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

In figure 13 shows a comparison between Ccon, HD and HDGT for all cancer-related morbidity endpoints. Kaplan Meier and Generalized Wilcoxon analysis indicates that there is a significant difference between the survival time for Ccon, HD and HDGT all cancer-related morbidity ($p>0.0001$).

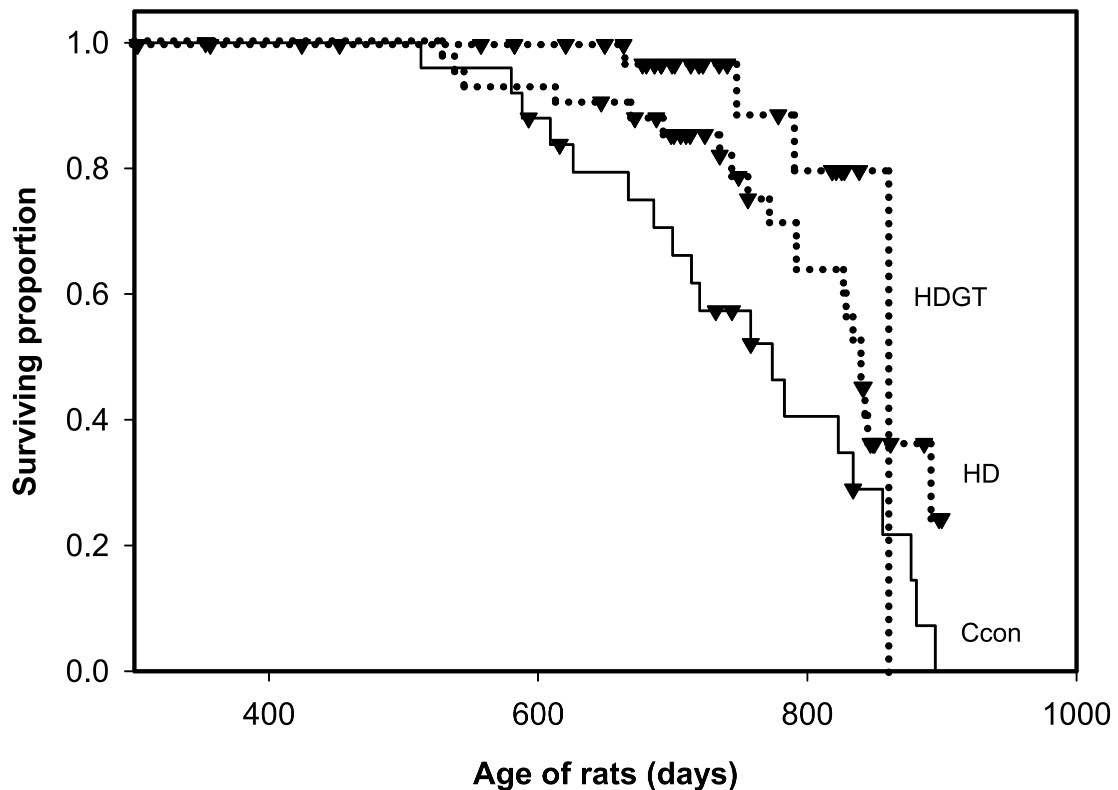

Figure 13: A comparison between Ccon and HD and HDGT for all cancer-related deaths (p>0.0001). All non cancer-related morbidity is shown as a censored data point illustrated by ▼. The survival times for CON and CGT were not statistically different and therefore their survival data has been combined into on group Ccon. Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

As in previous analyses it is necessary to examine the comparison of Ccon and the hot dog groups individually in order to determine the exact relationship between Ccon and the hot dog groups. A comparison between Ccon and HD for all cancer-related morbidity endpoints can be seen in figure 14.

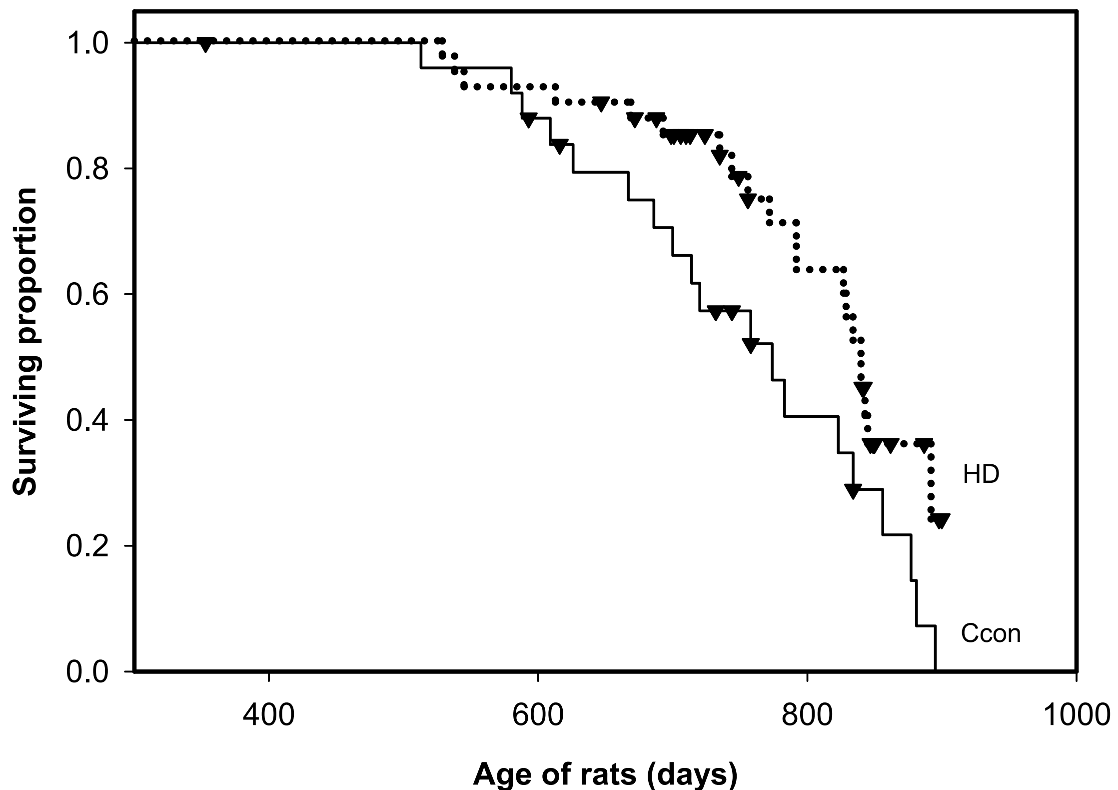

Figure 14: A comparison of survival time for cancer-related morbidity endpoints for Ccon and HD (p=0.975). All non cancer-related morbidity is shown as a censored data point illustrated by ▼. The survival times for CON and CGT were not statistically different and therefore their survival data has been combined into on group Ccon. Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

In figure 14 shows a comparison in survival time for cancer-related morbidity of Ccon and HD. Kaplan Meier and Generalized Wilcoxon analysis indicate that there is no significant difference the two groups (p=0.975). Figure 15 illustrates a comparison between survival time of Ccon and HDGT.

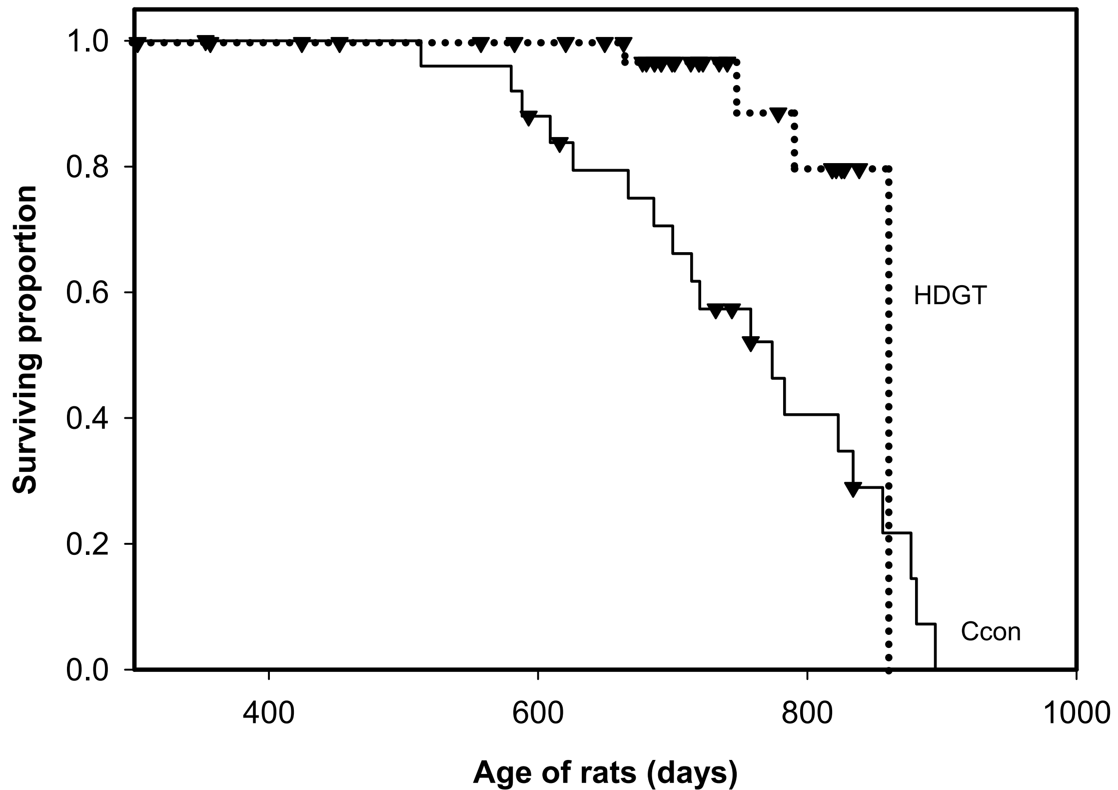

Figure 15: A comparison of survival time of Ccon and HDGT for all cancer-related morbidity (p=0.003). All non cancer-related morbidity is shown as a censored data point illustrated by ▼. The survival times for CON and CGT were not statistically different and therefore their survival data has been combined into on group Ccon. Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

Kalpan Meier and Generalized Wilcoxon test indicates a statistically significant difference in the survival time of Ccon for all cancer-related morbidity compared to the survival time for the HDGT rats (p=0.003).

Morbidity related to LGL leukemia

Histopathology findings indicated that many of the control animals had LGL leukemia whereas the incidence of LGL leukemia appeared to be lower in the hot dog rats compared to the control animals. As a result the potential effect of diet on the incidence of LGL leukemia warrants closer scrutiny. The following comparisons are LGL leukemia endpoints only and all other causes of morbidity have been censored from the data (this is denoted by an inverted triangle on the graph). A comparison of leukemia data from all diets can be seen in figure 16. When only leukemia related endpoints were considered there was no statistical difference between treatment groups for survival time (p=0.08).

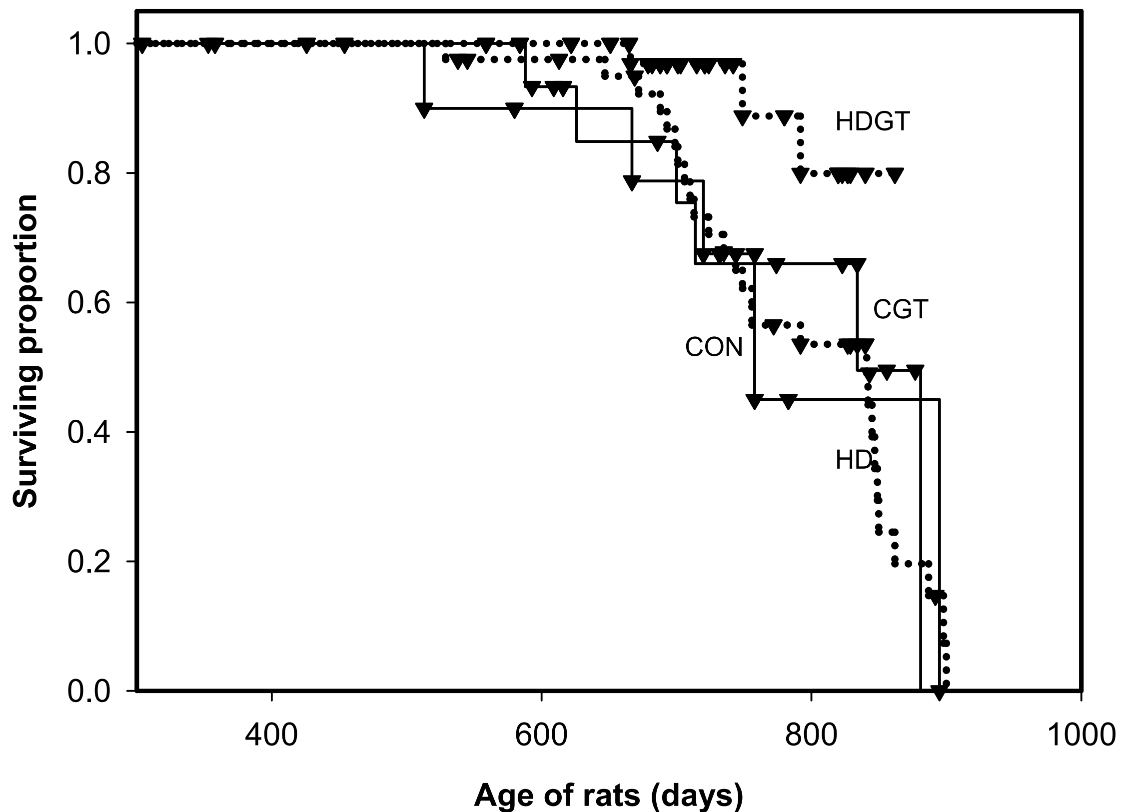

Figure 16: The survival time for rats with LGL leukemia. Survival times between groups were not statistically significant (p=0.08). All non leukemia related morbidity is shown as a censored data point illustrated by ▼. Diets (CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

As with the non censored data it was important to examine the relationship between each group as compared to CON. However as stated previously we will be attempting to combine CON and CGT into one group for the purposes of increasing statistical power. Determining whether there is any statistically difference between the two control groups will allow us to consider combining out control groups into one group. Figure 17 shows a comparison between CON and CGT.

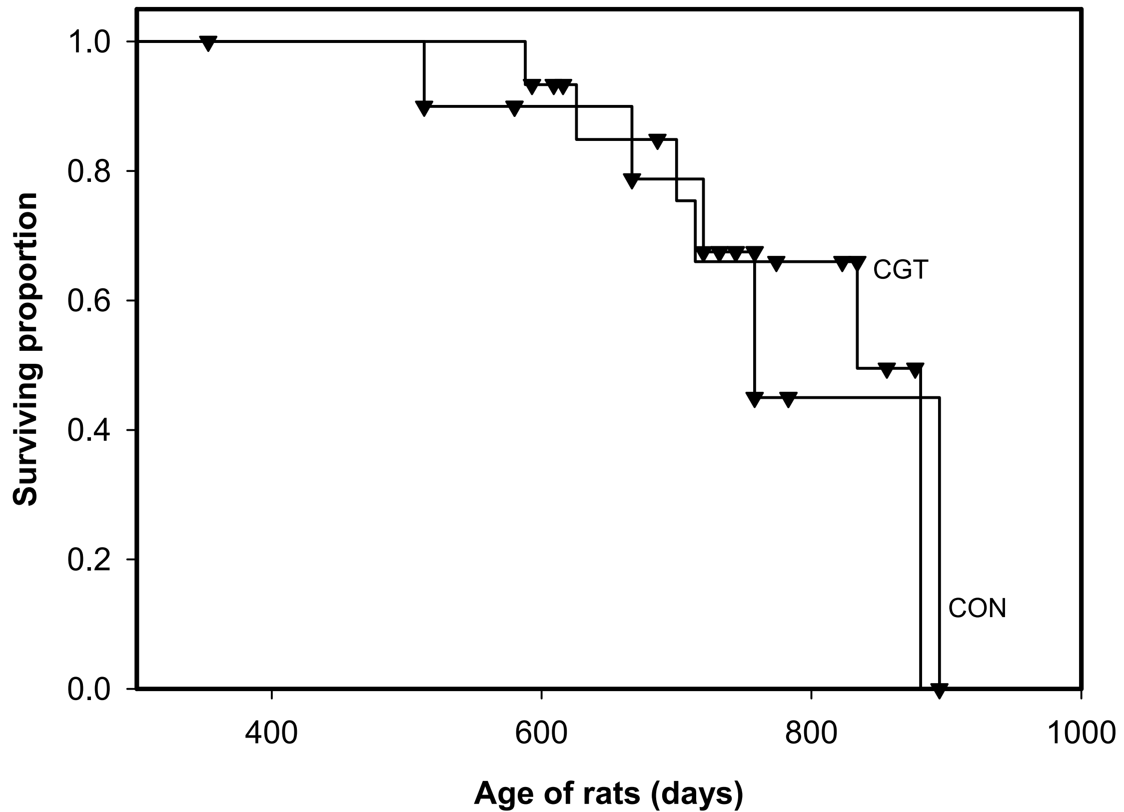

Figure 17: the effect of consumption of green tea catechins on the survival time of rats with LGL leukemia (p=0.782). All non leukemia related morbidity is shown as a censored data point illustrated by ▼Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

Figure 17 illustrates the comparison of leukemia related survival time of CON and CGT. There was no statistical difference between CON and CGT in the censored comparison (p=0.782). This result indicates that the addition of 0.5% green tea catechins to the control diet had no effect on the incidence of leukemia. This finding allows for the combining of the CON and CGT group as one combined control group. This will allow us to have a greater number of animals in our control group which in turn will provide a greater opportunity for statistical analysis to successfully examine potential diet effects. The following figure shows a comparison between Ccon (Combined control) and the hot dog groups.

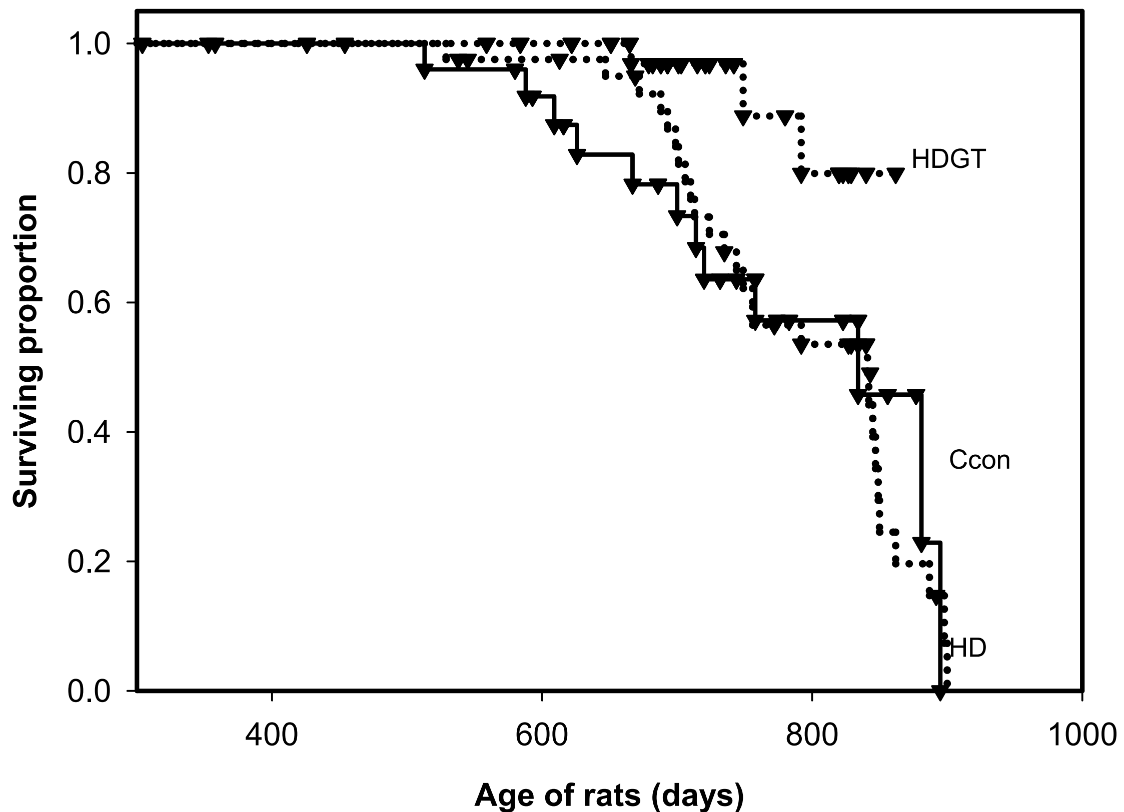

Figure 18: The effect of consumption of hot dog on the survival time of rats with LGL leukemia p=0.008. All non leukemia related morbidity is shown as a censored data point illustrated by ▼. The survival times for CON and CGT were not statistically different and therefore their survival data has been combined into on group Ccon. Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

Figure 18 illustrates the survival curve for combined controls and hot dog fed rats. Survival time for Ccon, HD and HDGT was statistically different for leukemia related morbidity (p=0.008). To determine where the statistical difference is located the following analysis compares the survival time for Ccon, HD and HDGT separately as pairs. In figure 19 shows a comparison between Ccon and HD.

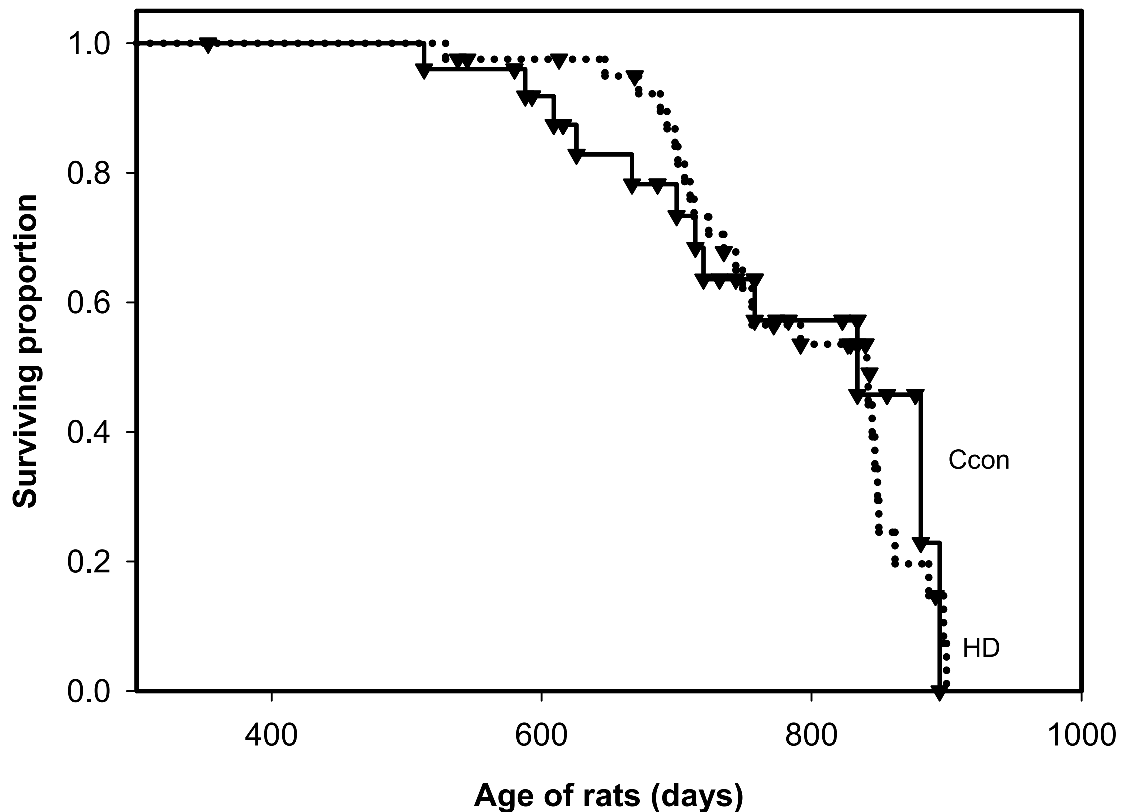

Figure 18: A comparison of survival time between Ccon and HD for all leukemia related morbidity p=0.162. All non leukemia related morbidity is shown as a censored data point illustrated by ▼. The survival times for CON and CGT were not statistically different and therefore their survival data has been combined into on group Ccon. Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

In figure 18 we see a comparison of the survival time between Ccon and HD. Kaplan Meier and Generalized Wilcoxon analysis indicates that there is no significant difference between the survival time of rats consuming HD when compared to the Ccon group (p=0.162).

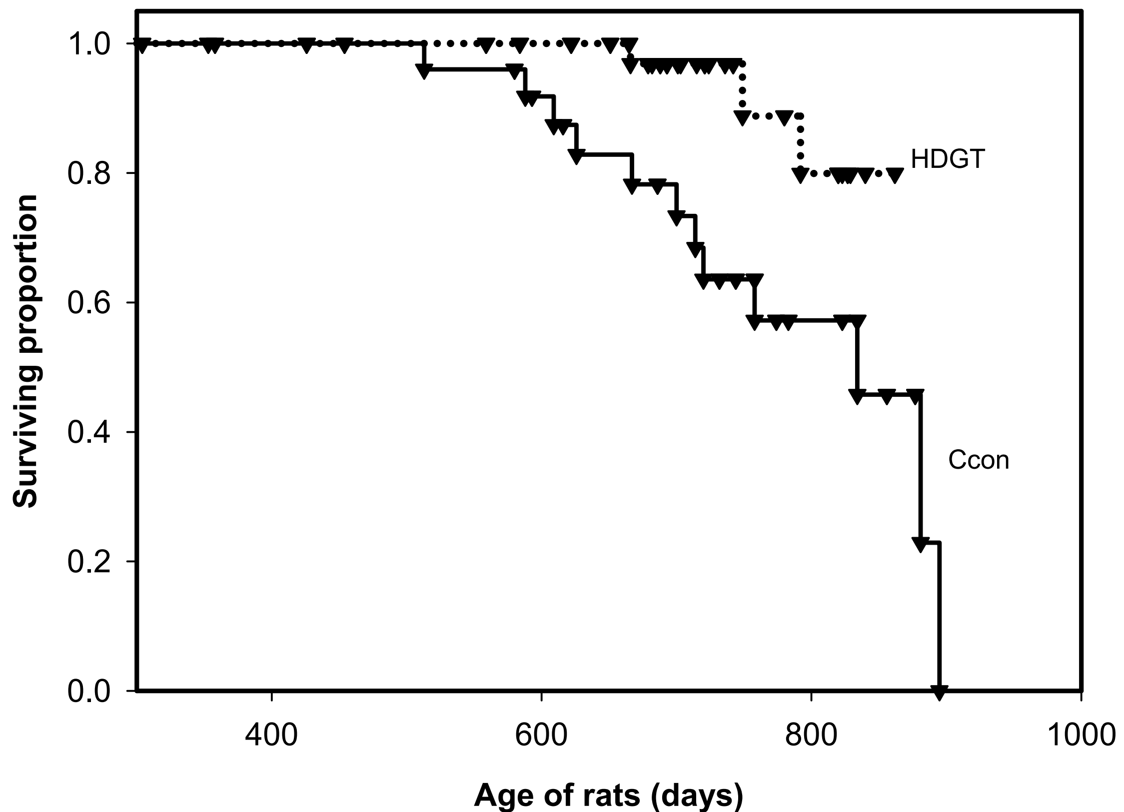

Figure 19: A comparison of survival time between Ccon and HDGT for all leukemia related morbidity p=0.011. All non leukemia related morbidity is shown as a censored data point illustrated by ▼. The survival times for CON and CGT were not statistically different and therefore their survival data has been combined into on group Ccon. Diets (CON (a modified AIN 93g diet), CGT(control diet +0.5% green tea catechins; HD(hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients). Diets were fed starting at 21 day of age and finishing when predetermined cancer morbidity endpoints were reached.

The survival time of rats with LGL leukemia was significantly greater for rats consuming HDGT than those of the Ccon group p=0.011 (figure 19).

The lifetime consumption of cooked and cured meat is reported to be a significant risk factor in increasing the likelihood of developing gastrointestinal cancer. In the data reported in this chapter we see that the consumption of hot dog throughout the lifetime of a rat does not induce gastrointestinal cancer. In addition, we see a significant lengthening of the life of rats fed hot dogs containing green tea catechins when compared to control animals. This indicates that consumption of hot dogs containing green tea may play a role in protecting DNA from forms of carcinogenesis. This will be addressed in more detail in the discussion section of this chapter. This potential role of catechin containing hot dogs may also play a role in preventing the rat from developing LGL leukemia, a type of cancer naturally formed during the later stage of life in F344 rats. The potential protective effect of hot dogs containing catechins appears to be missing from both the HD and CGT groups, indicating that in order to get the potential benefit of green tea it may have to be consumed in conjunction with a pro-carcinogen such as cooked and cured meat.

Discussion

Morbidity related to all cancers

The purpose of this study was to examine the effect of lifetime consumption of cooked and cured meat on the incidence of gastrointestinal cancer in F344 rats. Furthermore we examined whether the addition of green tea catechins to hot dogs could alter the incidence of gastrointestinal cancer in rats. Lifetime consumption of green tea catechins had no overall effect on rat survival in this experimental design. In addition, lifetime consumption of cooked and cured meat with or without the addition of green tea catechins had no overall effect on survival.

There is a substantial amount of epidemiological evidence suggesting that consumption of cooked and cured meat is associated with colorectal cancer in humans(2). In addition there is some evidence linking consumption of cooked and cured meat with esophageal and stomach cancers(2). As a result, with an intake of cured, grilled meat as 50% of all DM intake, we expected to see a significant number of intestinal adenomas in the HD fed rats. In this experiment we hypothesized that the addition of green tea to hot dogs would attenuate the carcinogenic quality of the hot dog. Green tea has been shown to prevent N-acetoxy-PHIP adducts in colon, heart, liver and lung(89) and B[a]P related adducts in liver(90) as well as endogenous nitrosamine formation(91). As a result we expected to see fewer intestinal cancers in the HDGT fed rats. Consequently the lack of dietary effect on survival time in all groups was unexpected.

One of the interesting aspects of this experiment was the apparent extended lifespan of the Fisher 344 rats. The estimated life span of F344 rats is approximately 18 months to 2 years of age (92). Other studies using caloric restriction to extend lifespan have reported rats living up to 36 months of age (93). Many of the rats in this experiment did not reach morbidity endpoints until well into their 3rd year of life. This old age resulted in our rats having health challenges such as weight loss attributed to age related muscle atrophy and pain related to age related joint degeneration. This may have resulted in rats being euthanized for the weight loss endpoint that was not cancer-related.

The consumption of green tea catechins alone did not affect cancer-specific morbidity. This result is somewhat unexpected as high green tea intake has been associated with lower risk of esophageal(94), stomach(71), colorectal(95) and liver(96) cancers. However, on a purified diet, there is limited exposure to dietary carcinogens and the tendency in these groups to develop gastrointestinal cancer was not predicted to be high. There has also been some evidence recently that rats and mice show an adaptive response when consuming a similar dose of green tea over 28 days. The rodents had an initial increase in plasma EGCG levels which was then followed by a decline in these levels(97). Similar results have been shown in humans(78). Even though plasma levels declined after repeated administration the level of EGCG was still present in the large intestinal tissue and esophagus of rats who consumed EGCG for 8 days(97). Therefore plasma levels of EGCG may not be a good measure of intestinal epithelial EGCG content.

Lifetime consumption of cooked and cured meat did not alter morbidity in F344 rats with cancer. Epidemiological studies have shown that there is significant evidence linking consumption of cooked and cured meat with colorectal cancer in humans(2). As a result it was unexpected that lifetime consumption of cooked and cured meat at 50% of DM intake did not cause gastrointestinal cancers and had no effect on all cancer-related morbidity. There have been conflicting results reported in the literature on long term consumption of processed meat. When Wistar rats consumed canned meat containing sodium nitrite in a long term study lasting 29 months no significant preneoplastic changes were seen in the colon (40). However in another study F344 rats fed cooked, processed meat containing sodium nitrite resulted in a significant increase in aberrant crypt foci in rat colon following injection with a known carcinogen azoxymethane (98). A similar model found an increase in stomach and colon cancer in rats treated with the carcinogen1,2-dimethylhydrazine and fed well cooked beef (99). In animal studies that have found a significant increase in colorectal cancer following consumption of meat the animals have also been injected or dosed with a known carcinogen. This does result in an increase in preneoplastic lesions and tumour formation; however, this may not be a realistic representation of the carcinogenic nature of cooked and cured meat. In articles where there is no underlying carcinogen being given there are no reported effects from consuming meat. It is possible that in healthy animals with no underlying xenobiotic stress or genetic predisposition that the consumption of cooked and cured meat does not result in a significant increase in cancers of the gastrointestinal tract.

The consumption of cooked and cured meat containing 1% green tea catechins resulted in a significant reduction in survival time for rats with cancer. This result was not expected. Our hypothesis states that the inclusion of green tea catechins would attenuate the carcinogenic qualities of PAH, HCA and NA present in the hot dog. Therefore hot dog containing green tea catechins should reduce morbidity seen in rats with cancer. It is important to note that only 7% of HDGT fed rats had cancer where as 73% of the Ccon group had cancer. Thus we are comparing the survival of 4 HDGT rats to the survival of 19 control rats. Differences between these groups could be exaggerated because of the vastly different sample sizes. Another possible cause for this dietary effect is that when green tea catechins were added to the hot dog it resulted in an obvious change in the chemistry of the meat. The mixture became stiff and dryer than the regular hot dogs. In addition, during the grilling of the hot dogs the green tea hot dogs tended to dry faster and shrink more than the regular hot dog. The addition of the catechins to the hot dog appeared to change the water holding capacity of the hot dog resulting in a dryer product. The dryer green tea hot dog could have resulted in an increased content of HCA and possibly PAH. This in turn would result in the HDGT rats being exposed to more carcinogens thus accounting for the significant reduction in survival of the HDGT rats with cancer. One of the main reasons why green tea catechins were chosen for this project was because of their heat stability and their ability to be mixed with protein without interacting with that protein. Research has shown that green tea mixed with milk does not alter the efficacy of the flavonoids (100). Consequently we did not anticipate the

catechin induced changes seen in the chemistry of the hot dog. In future studies microencapsulating the green tea catechins may improve the food chemistry challenges seen in this study.

Morbidity related to LGL leukemia

As the rats aged and began to reach morbidity endpoints, they were euthanized and necropsied. During necropsy a large number of rats were found to have splenomegly, and enlarged lungs and liver. Large granular cell leukemia (LGL leukemia) in rats is characterized by splenomegly, and infiltration of the spleen, lungs and liver by leukemic cells. As F344 rats age approximately 70% of control animals will develop LGL leukemia(101). In this experiment 86% of our Ccon rats had LGL leukemia. However our HD and HDGT rats did not display this high incidence of LGL leukemia.

Lifetime consumption of green tea had no significant effect on the survival of rats with LGL leukemia. ECGC has been shown to induce apoptosis *in vitro* in human leukemia (HL-60) cells(102), so there is some potential for an impact on this type of cancer. This mechanism may not be effective with LGL leukemia cells or the circulating ECGC maybe too low to induce apoptosis.

Lifetime consumption of hot dog did not affect the survival of rats with LGL leukemia. Processed meat consumption has been correlated with childhood leukemia in certain small case:control studies, but the data are not widely

accepted(103;104). In contrast, lifetime consumption of hot dog containing catechins resulted in a significantly longer survival time for rats with LGL leukemia. The consumption of catechins without the addition of hot dog did not have any effect on the occurrence of LGL leukemia. This indicates that in order to get the potential benefit of catechins they may have to be consumed in conjunction with a pro-carcinogen such as cooked and cured meat. HDGT rats had a significantly lower incidence of LGL leukemia when compared to Ccon. Therefore the consumption of HDGT conferred a level of protection to F344 rats against their genetic predisposition to developing LGL leukemia. It has been well noted in the literature that some chemicals which are detrimental at high doses result in stimulatory effects that are protective at lower doses (105). This effect has been seen in short term mechanistic studies and long term survival studies. Maekawa et al. found that rats consuming either 0, 2.5% or 5% sodium nitrate in drinking water had a significantly lower incidence of LGL leukemia at both the 2.5% and 5% treatment groups(106). As a result the lifetime consumption of sodium nitrate in hot dogs may have a similar effect. The lifetime consumption of HD or HDGT may result in an upregulation of DNA repair mechanisms which then in turn are more able to repair errors in DNA that would otherwise result in LGL leukemia.

An additional possibility is that lifetime consumption of nitrate may not be as harmful as previously believed. Dietary nitrate also has well defined health benefits(107). Dietary nitrate can act as a precursor for the synthesis of nitric oxide (NO)(108). NO is made in the endothelial cells lining the vascular vessels

and is responsible for vascular homeostasis and smooth muscle relaxation(108). Dietary nitrite can be used to replace endogenous NO production in endothelial nitric oxide synthase (eNOS) knockout mice. In this ischemia/repurfusion experiment dietary nitrate was fed to eNOS knockout mice. The dietary nitrite significantly reduced the amount of injury seen during the ischemia and subsequent reperfusion(108;109). In addition to sparing eNOS production of NO, dietary nitrite may play a role in reducing platelet aggregation and blood pressure(108). Dietary nitrite can be converted to nitrate in the body. Endogenous nitrate production can also be used to form nitric oxide. As a result, the sparing and potentially enhancing the levels of NO may play a valuable role in cellular health. Studies report that NO has a cytotoxic effect on tumours (110). Consequently it is possible, that dietary nitrates contained in hot dogs play an indirect role in tumour death.

The main finding of this experiment is that long term consumption of cooked and cured meat does not cause gastrointestinal cancer in F344 rats. This finding highlights the possibility that the rat may not be the best animal model for investigating the initiation of gastrointestinal cancer.

Chapter 3: The effect of co-consumption of cooked/cured meat and green tea catechins on phase 1 and phase 2 xenobiotic metabolisms in gastrointestinal tissues of male F344 rats

Abstract

Long term consumption of cooked and cured meat has been associated with increased risk of gastrointestinal cancer. Consumption of green tea catechins, namely EGCG, has been associated with prevention of gastrointestinal cancers. Ninety male F344 rats were weight matched and placed on either control (CON), control containing green tea catechins (CGT), hot dog fed with a nutrient supplement (HD), hot dog containing green tea catechins (HDGT) or hot dog fed with vitamin and mineral supplement containing green tea catechins (PH). Rats were fed diets for 21 days. Rats were then euthanized and esophagus, stomach, small intestine, large intestine and liver were harvested and microsomal fractions were prepared. Western blots for AHR, CYP1A1/A2, EROD assay and 2E1 were performed on cytosolic and microsomal fractions respectively. GST and UGT assays were conducted on cytosolic and microsomal fractions respectively. Consumption of hot dog resulted in upregulation CYP1A1/A2 and 2E1 in some gastrointestinal tissues and green tea catechins inhibited this upregulation in some tissues. Consumption of hot dog resulted in upregulation in UGT and GST in some gastrointestinal tissues and green tea catechins also caused upregulation in some tissues. Feeding green tea catechins may have resulted in an increase in UGT and GST in most gastrointestinal tissues. However this increase was not significant.

Introduction

Recent meta-analyses have examined the risk of developing gastrointestinal cancer associated with meat consumption. The consumption of red meat has been associated with colorectal cancer(2;3) and to a lesser extent esophageal and stomach cancer(2). Consumption of red meat that has been cooked and/or cured/ processed is associated with an increased risk of colorectal and gastric cancer(6;7;9-11;85;86). One proposed cause of this increased risk is the formation of heterocyclic amines (HCA), polycyclic aromatic hydrocarbons (PAH) and nitrosamines (NA) cooked and cured red meat.

HCA, PAH and NA are lipophyllic secondary carcinogens that are present in cooked and preserved meats such as sausages, hot dogs and smoked cold cut meats (19). Once ingested these molecules are metabolized to their carcinogenic forms by a process referred to as biotransformation or bioactivation. Biotransformation takes place mainly in the liver but also in the epithelium of esophagus, stomach, small intestinal and large intestine by CYP1A1/A2 and 2E1(41). The green tea catechin EGCG is thought to play an important role in inhibiting the upregulation of CYP1A1/A2 and CYP2E1 by competitively inhibiting AHR thus preventing further biotransformation of PAH, HCA and NA (48;82). Nitrite and amines react to form nitrosamines in foods or in the contents of the gastrointestinal tract, forming DMN, which is absorbed across the duodenal mucosa (38). DMN is bioactivated by CYP2E1 in the esophagus, small intestine or liver forming an electrophilic monoalkylnitrosamine and an aldehyde (45;60;61) . At this point GST may conjugate the monoalkylnitrosamine to a

glutathione molecule, making it stable and water soluble. If GST does not conjugate this reactive intermediate then it will rearrange and release a carbocation (carbanion). This is the ultimate carcinogen that can react with DNA bases creating various methylated bases, including 0^6methylguanine (19).

HCAs , such as IQ and PHIP, are bioactivated by CYP1A1/2 catalyzed oxidation of the exocyclic amine group of HCA into a reactive hydroxylamine derivative (54). Hydroxylamine is O-acetylated by NAT2 creating an N-acetoxy derivative which then undergoes heterocyclic cleavage creating a nitrenuim ion (22;55). The nitrenuim ion is reactive towards DNA and binds to the N2 and C8 atoms of guanine creating adducts. SULT metabolism of N-hydroxy derivatives can also form ultimate carcinogens from HCA(56). GST enzymes will metabolize electrophilic N-hydroxy derivatives. UGT conjugates either the exocyclic or imidazole nitrogen atoms of the N-hydroxy derivative making it water soluble and allowing for excretion(54).

PAH molecules and metabolism are highly variable, and most of the research is conducted on BaP as a model PAH. BaP is bioactivated by CYP1A and CYP1B to form an electrophilic epoxide in the 7,8 position. This epoxide is hydrolyzed by epoxide hydrolase (EH) forming a 7,8-diol. This 7,8-diol is further metabolized by CYP1A1/2 to a electrophillic 7,8-diol-9,10-epoxide, which is the ultimate carcinogen. PAH diol-epoxides react with nucleophilic sites on DNA forming adducts, leading to adducts on N^2-deoxyguanosine, N^7-deoxyguanosine and N^6-

deoxyadenine (57). The majority of BaP is metabolized by P450, GST and UGT in the liver, but intestinal xenobiotic metabolism also plays a role in PAH metabolism (58). Once the BaP diol-epoxide is formed, the nucleophilic diols may be used as sites for glucuronidation, while the electrophilic epoxide is conjugated very slowly by GST, due to stearic hindrance of the bay region(111-113). These slow reactions allow time for the diol-epoxide to form adducts with DNA.

EGCG has been shown to upregulate UGT and GST expression(36;36;114). Upregulation of UGT and GST results in an increase in the metabolism nucleophillic and electrophilic metabolites of PAH, HCA and NA thus facilitating their elimination. EGCG causes the upregulation of UGT and GST by inhibiting the dimerization of AHR with the aryl hydrocarbon receptor nuclear translocator thus inhibiting the upregulation of CYP1A1 and 1A2 and 2E1 (48;82).

EGCG may also interact with NRF2 by facilitating the phosphorylation of the serine and threonine residues NRF2 which causes its translocation to the nucleus where it binds to ARE thus resulting in UGT being upregulated (52). Upregulation of UGT would result in an increased ability to conjugate any nucleophilic metabolites of PAH and HCA, thus facilitating their elimination.

Our objective for the following experiment is to determine whether the consumption of cooked/cured meat results in the upregulation of cytosolic AHR, CYP1A1/A2 and 2E1. In addition, we hypothesize that the co-consumption of cooked/cured meat and green tea catechins will upregulation of GST and UGT and inhibit the upregulation of CYP1A1/A2.

Methods

Animals

Ninety male F344 rats weighing 50-75 g were purchased from Charles River, Canada. Animals were housed in environmentally enriched cages in accordance with the Animal Care Committee at the University of Guelph, Ontario, Canada. Wire bottom cages (20.50x26.25x14.5 cm) supplemented with environmental enrichment consisting of nesting boxes, newsprint paper, toys and wooden blocks. Upon arrival animals were weight matched and assigned to one of five treatment groups outlined below. Animals were fed assigned diets for 21 days. Health of the animals was monitored by weighing three times weekly weighing. To ensure ample tissue for all analysis 3 animals per treatment were pooled into one sample. This resulted in a sample size of 6 samples per treatment.

Diets

The 5 diets consisted of control (CON), control + 0.5% green tea catechins (CGT), hot dog (HD), hot dog containing 1% green tea catechins (HDGT) and hot dog + 1% green tea catechins (PH) (tables 6-9). The HD diet consisted of a 1:1 ratio of grilled pork hot dog containing 10% fat and a supplement containing vitamins, minerals and essential fatty acids added to meet the levels in AIN93g (87). The HDGT diet consisted of a 1:1 ratio of grilled 10% fat pork hot dog containing 1% w/w green tea catechins and the same supplement of vitamins,

minerals and essential fatty acids. The PH diet consisted of a 1:1 ratio of grilled pork hot dog containing 10% fat and a supplement containing 1% green tea catechins, vitamins, minerals and essential fatty acids added to meet the levels in AIN93g. Tables used to balance nutrients can be found in appendix 3. This diet was included to provide catechins in the portion of the diet that was not being cooked, for two reasons. When included in the hot dog, catechins could be degraded during cooking of the hot dog, and the presence of the catechins in the hot dog changed the water holding characteristics of the hot dog and changed the cooking properties of the hot dog. The CON diet was designed to provide the same levels of nutrients found in the HD diet, using casein, free amino acids and lard to replace the protein and fat of the hot dog component. The CGT diet was similar to the CON diet, with the addition of 0.5% w/w green tea catechins (Sunphenon 90LB; Taiyo Green power, Jiangsu, China; certificate of analysis in appendix 5). This level of catechins was designed to mimic the optimal dose of green tea in humans (estimated to be 10 cups/day or 1.5 litres) (69). This calculation used to convert human exposure to rat feeding is shown in appendix 2. All diets were mathematically balanced in the major nutrient classes and designed to be isocaloric. See appendix 3 for details on diet balancing and analysis.

Pork hot dogs were made by the Herman Laue Spice Company (Uxbridge, Ontario, Canada). The hot dogs contained 10% fat, used corn starch as a binder and 150 ppm sodium nitrite. Both types of hot dogs were smoked in a commercial smoker for 30 min at 155ºC. Hot dogs containing green tea

catechins had 1% w/w green tea catechins added to the hot dog mixture during the blending stage just prior to injecting the mixture into the casing and prior to the smoking stage. After smoking the hot dogs were packaged and frozen until use. Prior to feeding, both hot dogs and hot dogs containing catechins were grilled on standard counter top grills (Hamilton Beech) for 30 min at 350ºC. Grilled hot dogs were then frozen at -20ºC until needed. Hot dogs were thawed in the fridge prior to feeding. Animals were fed at a certain percentage of body weight in order to maintain even intake and growth among individuals. This percentage was adjusted downward over time to approximately 95% of *ad libitum* intake, so that all of the rats were consuming their allotment.

Table 6: Diet composition for control (CON) and control green tea diet (CGT). CON diet is based on AINg93 which has been balanced to match the nutrient content of the 10% fat pork hotdog + supplement containing vitamins, minerals and essential fatty acids. CGT diet is the control diet containing 0.5% green tea catechins. Diets were fed to rats for 3 weeks.

Ingredients	Control Diet (CON) % inclusion	Control + 0.5% green tea catechins (CGT) % inclusion
Cerelose	18.8	18.3
Casein	44.5	44.5
Celufil	5	5
Vitamin Mix	3	3
Mineral Mix	10	10
L-Cysteine	0.3	0.3
L_Histidine	0.5	0.5
L-Arginine	1	1
L-Lysine	0.7	0.7
Safflower oil	3.4	3.4
Flax oil	0.8	0.8
Lard	12	12
Green tea catechins	0.0	0.5
Total	100	100

Table 7: Diet composition for Hot dog (HD), Hot dog containing 1% green tea catechins (HDGT) and Hot dog with 1% green tea catechins in the supplement (PH). Hot dog diets were based on AINg93 which has been balanced to match the nutrient content of the 10% fat pork hotdog + supplement containing vitamins, minerals and essential fatty acids. HDGT diet consists of hot dog containing 1% green tea catechins. PH diet consists of the HD diet with 1% green tea catechins added to the powdered portion of the diet. Diets were fed to rats for 3 weeks.

Ingredients	Hot dog diet (HD) % inclusion	Hot dog diet containing 1% green tea catechins (HDGT) % inclusion	Hot dog diet fed with 1% green tea catechins (PH) % inclusion
Cerelose	32.3	32.3	32.3
Celufil	5	5	5
Vitamin Mix	1.5	1.5	1.5
Mineral Mix	5	5	5
L-Proline	2	2	2
Green tea catechin	0	0	1
Safflower oil	3.4	3.4	3.4
Flax oil	0.8	0.8	0.8
Grilled hot dog (Dry matter basis)	50	0	50
Grilled hot dog (1% green tea) (Dry matter basis)	0	50	0
Total	100	100	100

Table 8: Vitamin premixes for control and hot dog diets. The inclusion level for premix was 3% w/w for control diets and 1.5% w/w for hot dog diets.

Ingredients	Control Diet g/100g	Hot dog diet g/100g
Thiamin	0.0509	0.0000
Riboflavin	0.0214	0.0100
Nicotinic acid	0.2990	0.0000
Ca pantothenate	0.0530	0.0100
Pyridoxine	0.0270	0.0000
Folic acid	0.0120	0.0200
Vitamin B-12	0.0001	0.0001
Retinol	0.0004	0.0080
Vitamin D	0.0001	0.0001
Vitamin E (α-tocopherol)	0.1790	0.3580
Vitamin K (phylloquinone)	0.0030	0.0060
Choline	3.5600	7.1300
Biotin	0.0007	0.0010
Sucrose	95.7770	92.4000
Total	100.0000	100.0000

Table 9: Mineral premixes for control and hot dog diets. Control diets contained 10% w/w mineral mix and hot dog diets contain 5% w/w of their respective mix.

Ingredients	Control diet g/100g	Hot dog diet g/100g
Calcium carbonate	13.360000	26.29
Ferric Citrate	0.290000	0.172
Magnesium oxide	0.910000	0.782
potassium phosphate monobasic	16.220000	0.000
potassium citrate, tripotassium monohydrate	7.880000	0.000
Sodium chloride	5.850000	0.000
Zinc carbonate	0.070000	0.052
Cupric Carbonate	0.010000	0.026
Manganous carbonate	0.020000	0.052
Sodium selenate anhydrous	0.001000	0.000
Potassium Sulfate	1.740000	3.49
Potassium Iodate	0.000300	0.0007
Ammonium paramolybdate 4H20	0.000200	0.00059
Sodium meta-silicate 9H20	0.054000	0.108
chromium potassium sulfate 12H20	0.010200	0.02
Sodium fluoride	0.002000	0.004
Nickel carbonate	0.001000	0.002
Boric Acid	0.003000	0.006
Lithium chloride	0.000065	0.001
ammonium vanadate	0.000200	0.0004
Sucrose	53.510000	68.98
TOTAL	100.000000	100

Tissue harvesting

On day 21 of feeding animals were fasted for 14 hours overnight. The following morning (day 22) rats were anesthetized using 4% isoflourine (Baxter corporation, Mississauga, ON) and then decapitated. Immediately following decapitation esophagus, stomach, liver, duodenum (first 10 cm of small intestine) and large intestine were excised and rinsed in ice cold phosphate buffered saline containing EDTA (13.68 mM sodium chloride, 0.26mM potassium chloride, 1.52 mM sodium phosphate, 0.14 potassium phosphate monobasic, 1mM EDTA). Prior to homogenization large and small intestine were flushed and the stomach was opened along the greater curvature, contents were emptied and rinsed with PBS + EDTA. All tissues were homogenized on ice in 5mL of ice cold homogenization buffer containing 50 mM tris-HCl, 150 mM potassium chloride, 250 mM sucrose, 1 mM EDTA at pH 7.4. Just prior to use 1mM Pefabloc SC (AEBSF) and 3500 units/mL trypsin inhibitor (Gibco 179) was added to the buffer. All buffer ingredients are from Sigma-Aldrich, Oakville, ON, Canada unless otherwise stated. Polytron PT 1300 homogenizer with a 12mm head at 12 000 x g was used for all homogenizations. An s9000 fraction was then prepared using Sorvall RC 5C plus centrifuge at 8600 rpm (~9000g) using a for 30 minutes at 4°C. The resulting supernatant was saved. At this time the supernatants of 3 rats was combined into one sample per treatment to form a final sample size of six. Combined supernatants were then frozen in liquid nitrogen and stored at -80°C until microsome preparation.

Microsome and Cytosol Preparation

S9000 fractions were thawed on ice and then transferred to polycarbonate ultra centrifuge tubes (Beckman Coulter, Fullerton, CA, USA). Samples were then centrifuged for 60 minutes at 40 000 rpm (107 000 x g) at 4ºC using a Beckman Coulter Optima LE 80K ultracentrifuge. The supernatant (cytosolic fraction) was decanted into 1.5 mL tubes. A 10 µL aliquot of supernatant was retained for BCA protein assay. The remaining cytosol was frozen in liquid nitrogen and then stored at -80ºC. The remaining pellet was disrupted and then transferred from the ultracentrifuge tube into a glass teflon homogenizer. Microsomal buffer (pH 7.4) containing 50 mM Tris-HCl, 20% v/v Glycerol, 1 mM EDTA, 1mM Pefabloc SC, 3500 units/mL trypsin inhibitor (Gibco 179) was added to each pellet in the following volumes 500µL esophagus, 1mL stomach, 2mL liver, 1mL small intestine, 1mL large intestine. Differing volumes of buffer were used to account for differing weight of tissue. Samples were then homogenized (approximately 30 strokes) on ice. Microsomes were then transferred into two 1.5 mL tubes. A 10 µL aliquot of microsomes was retained for BCA protein assay. Microsomes were frozen in liquid nitrogen and stored at -80ºC.

Protein Quantification

All cytosolic and microsomal samples were analyzed for quantity of protein using BCA protein assay (bicinchoninic acid and 4% copper II sulfate) using BSA as a standard. Samples were diluted to ensure the results fit in the middle range of

the standard curve. 0.01% copper II sulfate in bicinchoninic acid was mixed with 25μL of each sample and then incubated at 37ºC for 30 min. The plate was then read at 562 nM on a Molecular devices spectramax plus, Sunnyvale, CA, USA).

Immuno-quantification of CYP P450 1A1/1A2, 2E1 and AhR receptor

Samples were diluted with 2x Laemmli loading buffer (125mM Tris base, 20% glycerol v/v, 4% SDS w/v, 0.006% Bromophenol Blue) to create a final working volume of 5 μg/μL for stomach, small intestine, large intestine and liver. A working volume of 1 μg/μL was used for esophagus. For all tissues except esophagus 100ug of protein per well was loaded onto 8% sodium dodecyl sulfate–polyacrylamide gels. 50 ug of esophageal protein was loaded onto 8% sodium dodecyl sulfate–polyacrylamide gels. Proteins were electrophoresed and then transferred to nitrocellulose membranes (Hybond-ECL, Amersham, Baie d'Urse, QC, Canada) using a wet transfer apparatus (Bio-Rad, Mississauga, Ontario). Even loading of protein was confirmed by staining membranes with Fast Green (1 mg/ml methyl green in 20% methanol, 5% acetic acid). Membranes were blocked in phosphate buffered saline containing 5% nonfat dry milk powder, and 0.1% tween 20 for 1 hour at room temperature and subsequently incubated with primary antibody overnight at 4ºC. Membranes were then washed and incubated with either a rabbit or mouse secondary antibody conjugated to horseradish peroxidase. Membranes were washed again and dipped in chemiluminescence (ECL) reagent (Pierce Thermo Fisher

Scientific, Ottawa, ON). Proteins were detected and quantified by densitometry (ChemiGenius2 Bioimaging, SynGene, Cambridge, U.K.).

Antibodies

All samples were probed using the following antibodies: A monoclonal primary antibody against CYP 450 1A1/1A2 (CD5.1) using a 1:1000 dilution. A polyclonal CYP 450 2E1 using a 1:400 dilution. Both CYP antibodies were purchased from Dr. Paul Thomas, Rutgers University, New Jersey, USA. Aryl hydrocarbon receptor monoclonal antibody (RPT1) was obtained from Abcam, Cambridge MA, USA, using a working dilution of 1:1000. Goat anti-rabbit conjugated to horseradish peroxidase and was used at a working dilution of 1:10 000 (Jackson Immuno Research, West Grove, PA, USA). Goat anti-mouse secondary antibody conjugated to horseradish peroxidase and was used at a working dilution of 1:20 000 (Jackson Immuno Research, West Grove, PA, USA).

EROD 96 well plate assay

For all tissue microsomes except esophagus 250 µg of protein/well was used for the assay. Due to the limited amount of esophageal protein only 100 ug of microsomal protein/well was used for the assay. Microsomal protein was diluted using EROD Assay Buffer (pH 7.8) containing 100mM HEPES and 5mM $MgCl_2 \cdot 6H_2O$. In each well 250 µg of protein was combined with 5 µM/well 7-ethoxyresorufin (dissolved in Dimethyl sulfoxide) and 2µM/well β-Nicotinamide

adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate. All reagents are from Sigma-Aldrich, Oakville, ON, Canada unless otherwise noted. The samples were read at time 0 with an absorption wavelength of 530nm and an emission wavelength of 590nm on a Bio-Tek Synergy HT fluorescence microplate reader (Winooski, VT, USA). Samples were then incubated overnight at 25ºC and read again 15 hours later.

EROD enzyme activity calculation

All calculations were done using the extinction coefficient of resorufin ($72 mM \cdot cm^{-1} \cdot L^{-1}$). The slope was calculated and then corrected for the amount of the blank slope. The corrected slope was then divided by the extinction coefficient. Path length was then corrected for by dividing by 0.25cm. The volume of the reagents in the well was then accounted for by multiplying by 0.0002L. The amount of protein in the well was then accounted for by dividing by the amount of protein/well in milligrams. Finally the result was converted from mM to nMol by multiplying by 1×10^6. The resulting enzyme activity is reported in nMol/min/mg protein.

UDP glucuronsyl transferase 96 well plate assay

Microsomal protein was used in this assay because UGT is a microsomal protein. For all tissue microsomes except esophagus and liver 100 μg of protein/well was used for the assay. Due to the limited amount of esophageal protein only 20 ug of microsomal protein/well was used for the assay. 20 ug of

microsomal protein/well for liver samples was used because of the high degree of UGT activity in this tissue. Reactions were carried out in 0.6 mL tubes containing the appropriate amount of protein diluted with a reaction buffer (317mM Tris-HCl, 47mM $MgCl_2$, 9mM Phenolphthalein (in 95% ethanol), 3% BSA, 3mM 2-Mercaptoethanol (pH 8.0)) and 7mM UDP glucuronic acid tri-sodium salt at 37ºC. Time 0 reactions were stopped by using 180mM glycine (pH 10.4) and then read at 552 nM on a Molecular devices spectramax plus, Sunnyvale, CA, USA. Remaining unstopped reactions were allowed to incubate for 2 hours at 37ºC. Reactions were then stopped and read on the spectrophotometer.

UGT enzyme activity calculation

All calculations were done using the extinction coefficient of phenolphthalein (26 $mM \cdot cm^{-1} \cdot L^{-1}$). The slope was calculated and then corrected for the amount of the blank slope. The corrected slope was then divided by the extinction coefficient. Path length was then corrected for by dividing by 0.25cm. The volume of the reagents in the well was then accounted for by multiplying by 0.0002L. The amount of protein in the well was then accounted for by dividing by the amount of protein/well in milligrams. Finally the result was converted from mM to nMol by multiplying by 1 $x10^6$. The resulting enzyme activity is reported in nMol/min/mg protein. The result was then converted from the disappearance of phenolphthalein to the appearance of the phenolphthalein glucuronide conjugate by multiplying by -1.

Glutathione S-transferase 96 well plate assay

Cytosolic protein was used for the GST assay because the majority of GST isomers are cytosolic proteins. For all tissue cytosol preparations except esophagus and stomach 4 µg of protein/well was used for the assay. Due to the limited amount of GST activity in esophagus and stomach 40 ug of cytosolic protein/well was used for the assay. Samples were diluted using HEGD buffer (25mM HEPES, 1.5mM EDTA, 10% glycerol, and 1mM DTT). In each well, the appropriate amount of protein was combined with 7.3mM phosphate buffer (10mM potassium phosphate monobasic and 10mM potassium phosphate dibasic, pH 6.5), 1mM L-glutathione reduced and 1mM CDNB (1-chloro-2,4-dinitrobenzene). The plate was then read immediately read at 340 nM on a Molecular devices spectramax plus, Sunnyvale, CA, USA. The samples were incubated for 20 minutes at room temperature and then read again to obtain the final absorbance.

GST enzyme activity calculation

All calculations were done using the extinction coefficient of CDNB (9.6 $mM \cdot cm^{-1} \cdot L^{-1}$). The slope was calculated and then corrected for the amount of the blank slope. The corrected slope was then divided by the extinction coefficient. Path length was then corrected for by dividing by 0.25cm. The volume of the reagents in the well was then accounted for by multiplying by 0.0002L. The amount of

protein in the well was then accounted for by dividing by the amount of protein in milligrams in each well. Finally the result was converted from mM to nMol by multiplying by 1 $x10^6$. The resulting enzyme activity is reported in nMol/min/mg protein.

Statistical analysis

All statistical analysis was performed using SPSS 16.0 statistical software package.   Normality of data was assessed using Levene statistic. Western blot data had a non normal distribution and thus data was analyzed using Kruskal-Wallis and Wilcoxon signed ranks tests. Growth and EROD assay data was analyzed using ANOVA and LSD (Least Significant Difference) post hoc test or Wallis and Wilcoxon signed ranks tests when data was not normally distributed. UGT and GST assays were analyzed using ANOVA and LSD post hoc test or Wallis and Wilcoxon signed ranks tests when data was not normally distributed. Normality was assessed using Levene statistic. Results were considered statistically significant if $p \leq 0.05$. Results were considered statistically a trend if $p \leq 0.10$.

Results of phase 1 xenobiotic metabolism

In this experiment our goal was to examine the effect of 21 days of dietary treatment (outlined in the methods) on the phase 1 xenobiotic enzymes. Rats were fed their appointed diet starting at approximately 25 days of age and continued for 21 days. Rats grew normally over the course of the experiment (figure 20). There was no statistical difference in the average body weights between the treatment groups ($p \geq 0.05$).

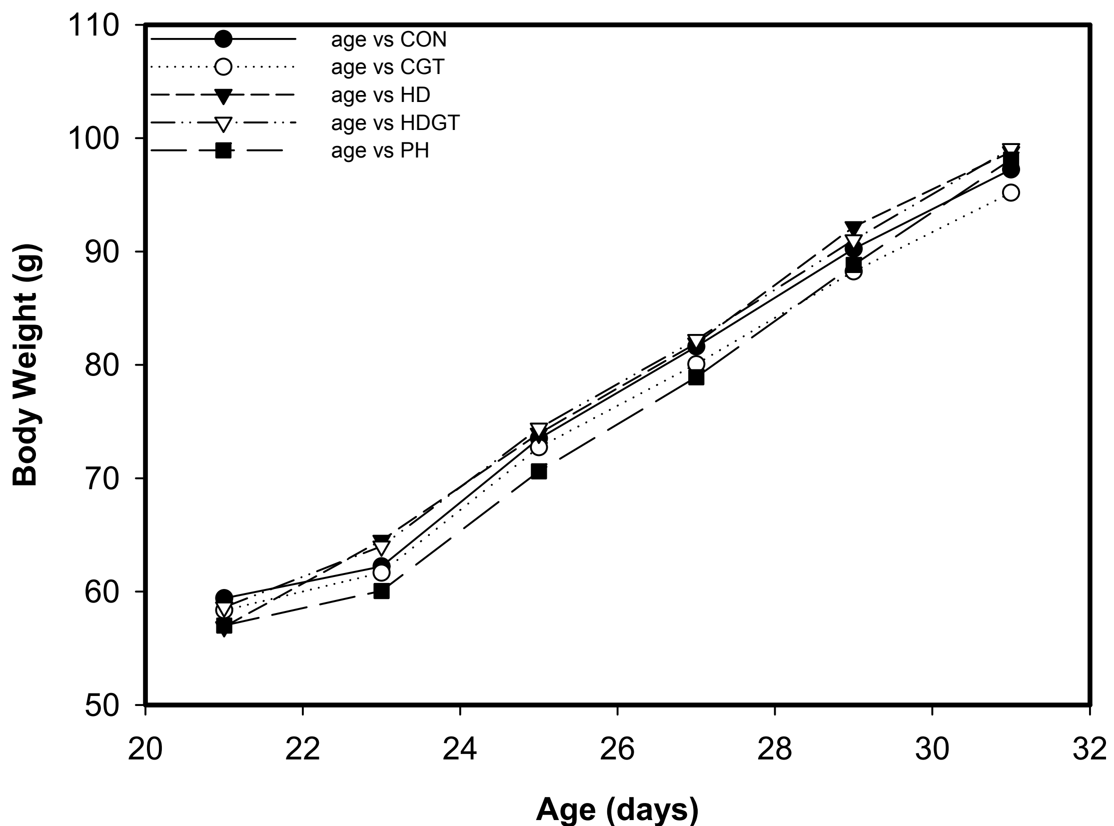

Figure 20: Increase in rat weight over time as an indicator of growth and general health. Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); HD (hot dog diet fed 1:1 with supplemented

nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients); or PH (hot dog fed with 1:1 supplemented nutrients containing 1% green tea catechins). Diets were fed for 21 days (n=6).

Immuno-quantification of CYP proteins and AhR

Proteins extracted from esophagus, stomach, liver, small intestine and large intestine were probed for CYP1A1/A2, CYP2E1 and AhR. The antibody used to quantify CYP1A1/2 does not differentiate between 1A1 and 1A2, but the results are reported based on the known tissue-specific expression of these isozymes. CYP1A1 and CYP1A2 can bioactivate HCA, PAH and NA so we can consider the activity of these CYPs together as one result(42;45).

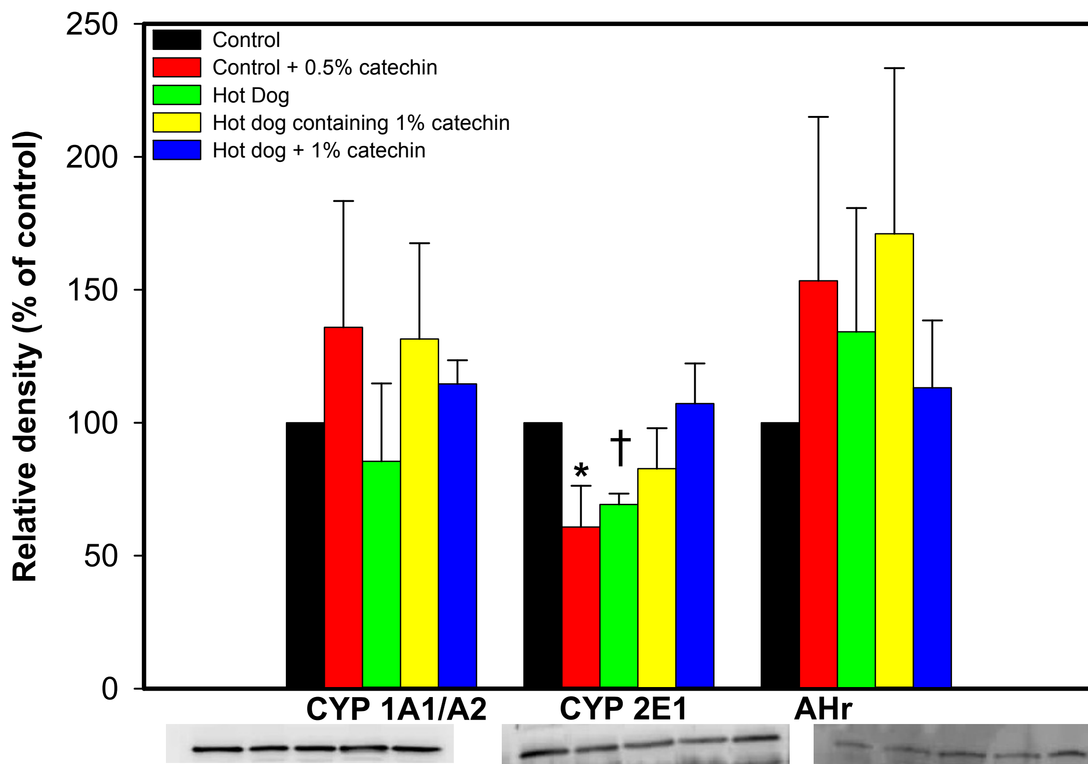

Figure 21: Western blot of esophagus microsomes (CYP1A1/A2) and cytosol (AhR). Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); HD (hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients); or PH (hot dog fed with 1:1 supplemented nutrients containing 1% green tea catechins). Diets were fed for 21 days (mean±SEM; n=6).

*** indicates a trend in catechin effect (p=0.075); † indicates a significant hot dog effect (p=0.028)**

In figure 21 we see the results for western blots of esophagus microsomes. Esophagus contains both CYP1A1 and 1A2 isoforms(45). There was no significant difference between treatment groups for CYP1A1/1A2. Rats fed HD had a significantly lower level of CYP2E1 when compared to CON fed rats ($p=0.028$). The comparison between CON and CGT is not significant ($p= 0.075$) however the p value could be considered a trend indicating that the addition of green tea catechins to rat diet decreased the level of CYP2E1 in the esophagus. There was no significant difference in AhR levels between treatment groups.

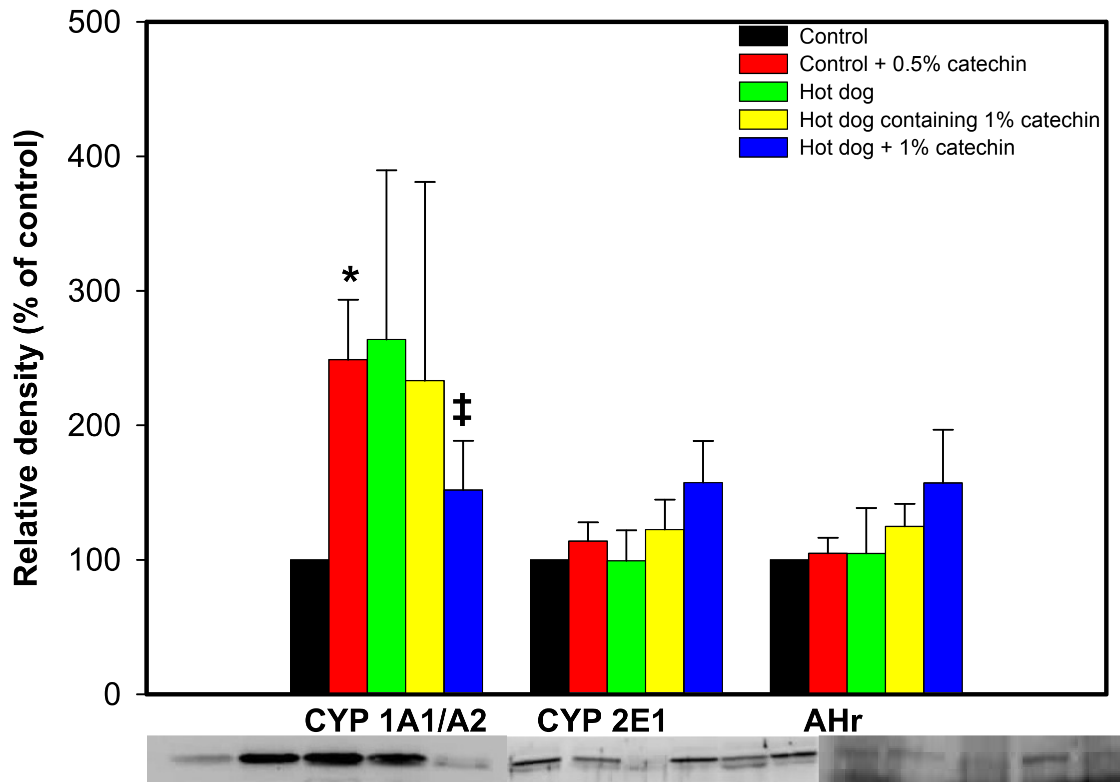

Figure 22: Western blot of stomach microsomes (CYP1A1/A2) and cytosol (AhR). Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); HD (hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients); or PH (hot dog fed with 1:1 supplemented nutrients containing 1% green tea catechins). Diets were fed for 21 days (Mean±SEM; n=6).

*** indicates a significant catechin effect (p=0.046); ‡ indicates a significant hot dog/catechin effect (p=0.046)**

Stomach contains both CYP1A1 and CYP1A2 (45). Figure 22 shows a significant increase in CYP1A1/A2 in the stomach of rats fed CGT diet when compared to CON ($p = 0.046$). PH fed rats also had a significantly higher level of CYP1A1/A2 in the stomach when compared to CON fed PH rats ($p=0.046$). The remaining diets did not cause a significant change in CYP1A1/A2 levels in the stomach. There was no significant difference in CYP2E1or AhR levels in rat stomach cytosol for any of the dietary treatments.

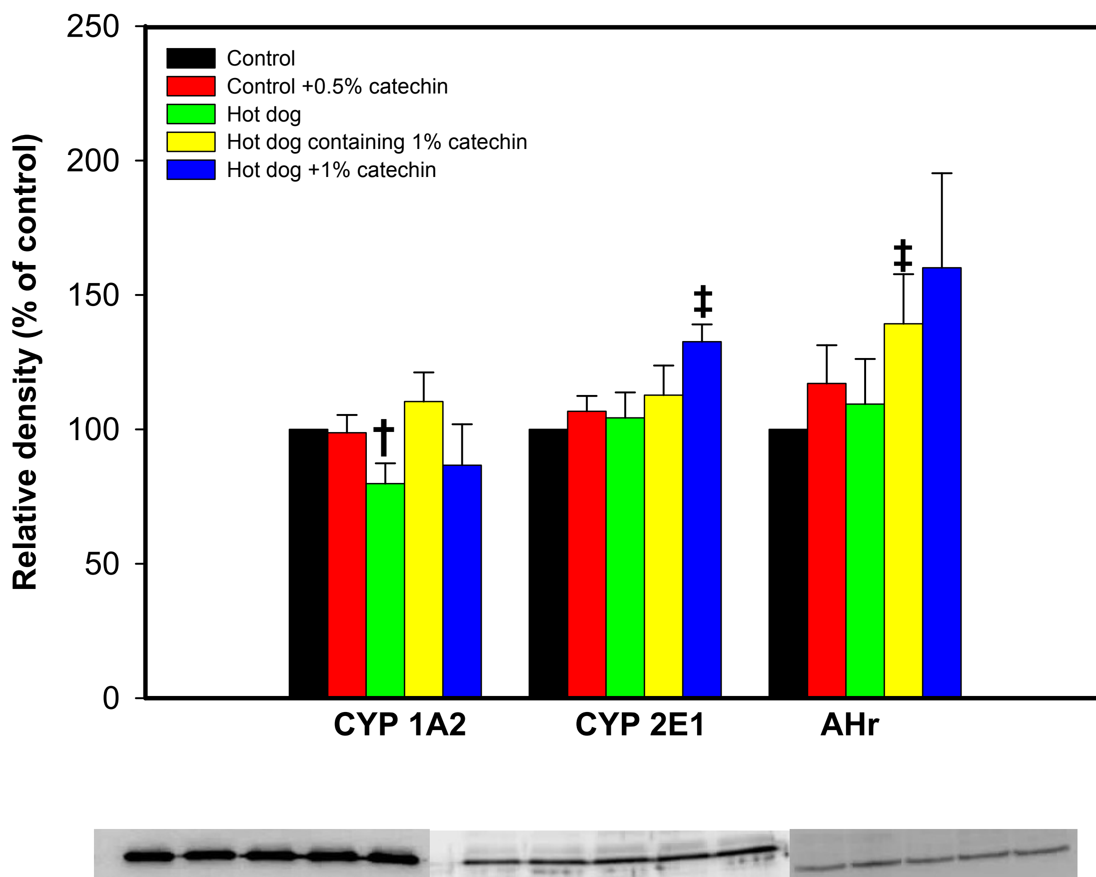

Figure 23: Western blot of liver microsomes (CYP1A1/A2) and cytosol (AhR). Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); HD (hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients); or PH (hot dog fed with 1:1 supplemented nutrients containing 1% green tea catechins). Diets were fed for 21 days (Mean±SEM; n=6).

† indicates a significant hot dog effect (p=0.028); ‡ indicates a significant hot dog/catechin effect (p=0.028); ‡ indicates a significant hot dog/catechin effect (p=0.046)

Liver contains only CYP1A2(45). Figure 23 shows a significant decrease in CYP1A2 levels in the liver microsomes of rats fed HD diet when compared to CON fed rats (p=0.028). The remaining diets did not cause a significant change in liver CYP1A2.   Rats fed the PH diet had a significant increase in liver CYP2E1 when compared to CON fed rats (p=0.028). Remaining diet had no significant effects on CYP2E1 levels. AhR was significantly increased in the liver cytosol of rats consuming HDGT diet when compared to CON fed rats (p=0.046). All remaining diets had no significant effect on liver AhR.

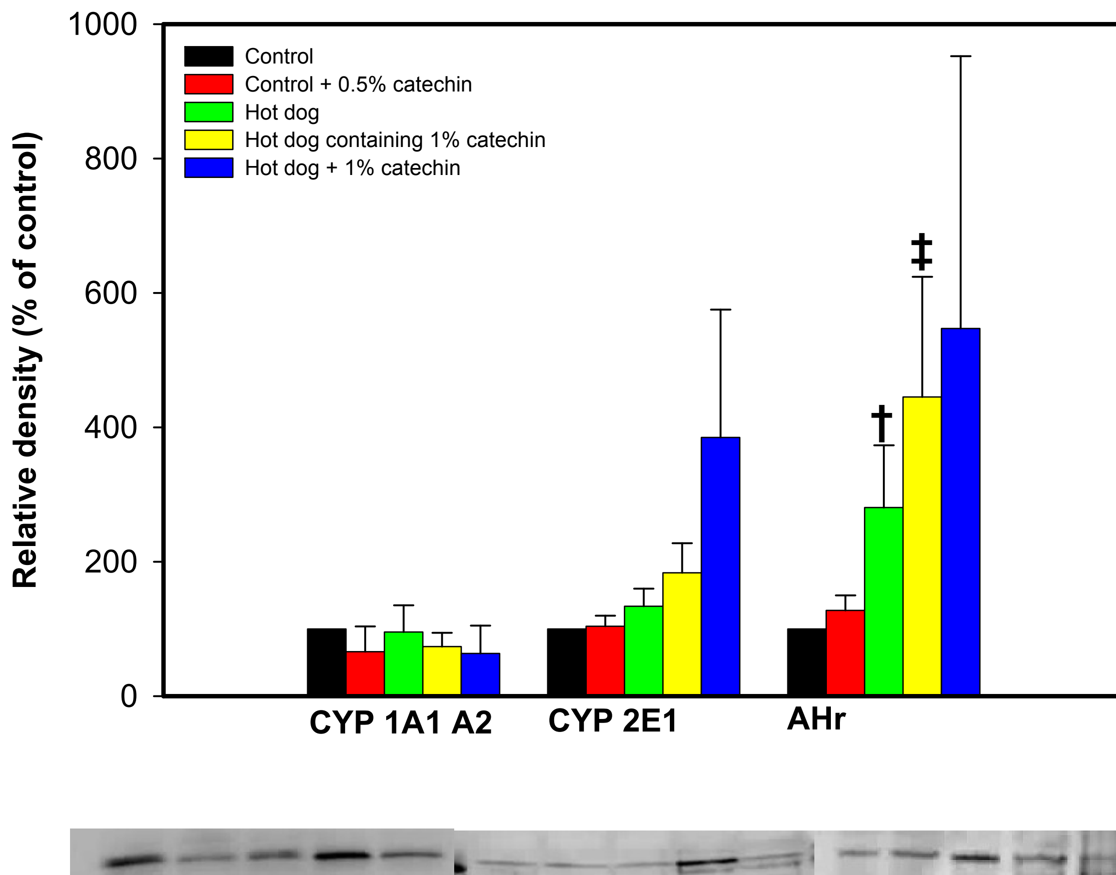

Figure 24: Western blot of small intestine microsomes (CYP1A1/A2) and cytosol (AhR). Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); HD (hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients); or PH (hot dog fed with 1:1 supplemented nutrients containing 1% green tea catechins). Diets were fed for 21 days (Mean±SEM;n=6).

† indicates a significant hot dog effect (p=0.028); ‡ indicates a significant hot dog/catechin effect (p=0.046)

Figure 24 illustrates a significant increase in AhR levels in the small intestine cytosol of rats fed HD when compared to CON fed rats (p=0.028). In addition this significant increase in AhR was also seen in the small intestine of rats fed PH diet (p=0.046). There was no significant difference seen in small intestinal cytosol AhR levels in the remaining diets. No significant difference was seen in small intestinal CYP1A1/A2 levels or CYP2E1 levels.

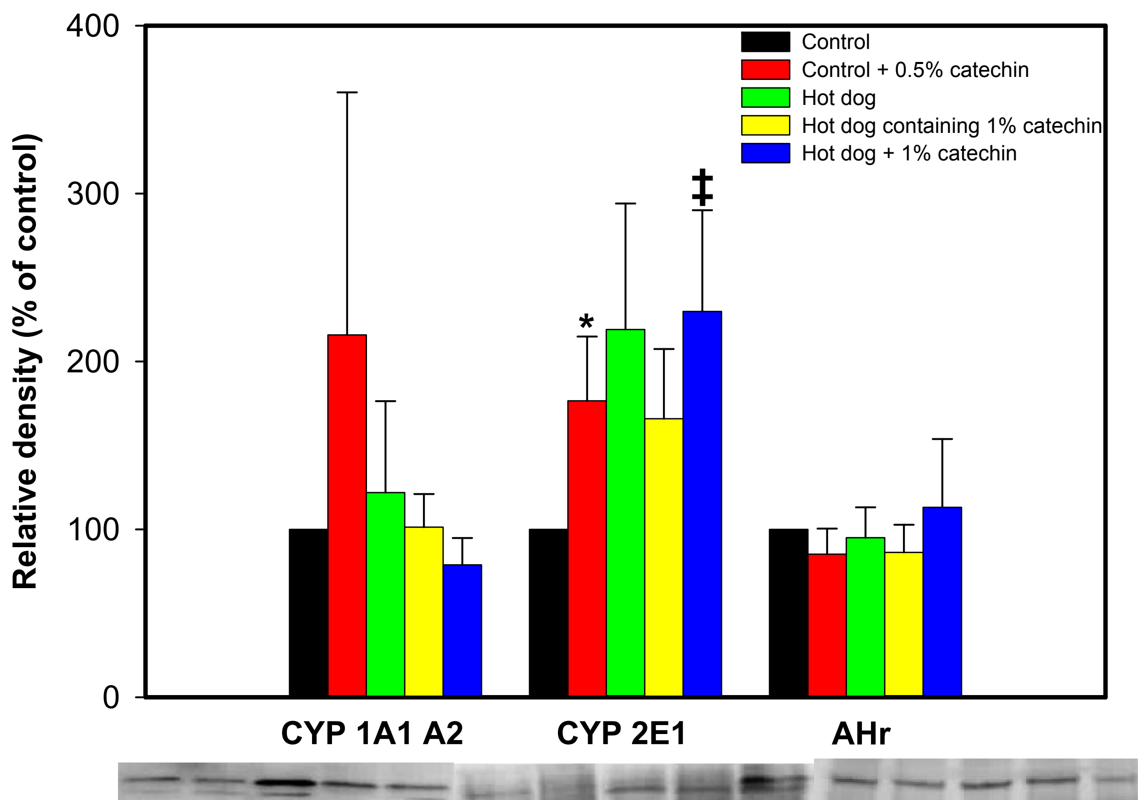

Figure 25: Western blot of large intestine microsomes (CYP1A1/A2) and cytosol (AhR). Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); HD (hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients); or PH (hot dog fed with 1:1 supplemented nutrients containing 1% green tea catechins). Diets were fed for 21 days (Mean±n=6).

*** indicates a significant catechin effect (p=0.028); ‡ indicates a trend in hot dog/catechin effect (p=0.075)**

In figure 25 we see a significant increase in large intestine CYP2E1 in rats fed CGT diet when compared to CON (p=0.028). This significant increase is also seen in large intestinal CYP2E1 in rats fed the PH diet (p=0.075). The remaining diets had no significant effect on CYP2E1 in rat large intestine. No significant difference was seen in large intestinal CYP1A1/A2 levels. In addition dietary treatments had no significant effect on large intestinal cytosol AhR levels.

EROD assay

The EROD assay was conducted on esophagus, stomach, liver, small intestine and large intestinal microsomes to provide a measure of CYP1A1/A2 catalytic activity. CYP1A1 and CYP1A2 both react with the substrate used in the EROD assay and consequently the results of this experiment consider CYP1A1 and 1A2 together when both isoforms are present. CYP1A1 and CYP1A2 can both bioactivate HCA and PAH, so it is logical to consider the activity of these CYPs together. Not all tissues reported below express both CYP1A1 and 1A2, therefore some EROD results will be for both CYP1A1 and CYP1A2 and other results will be for only CYP1A1 or CYP1A2 (45).

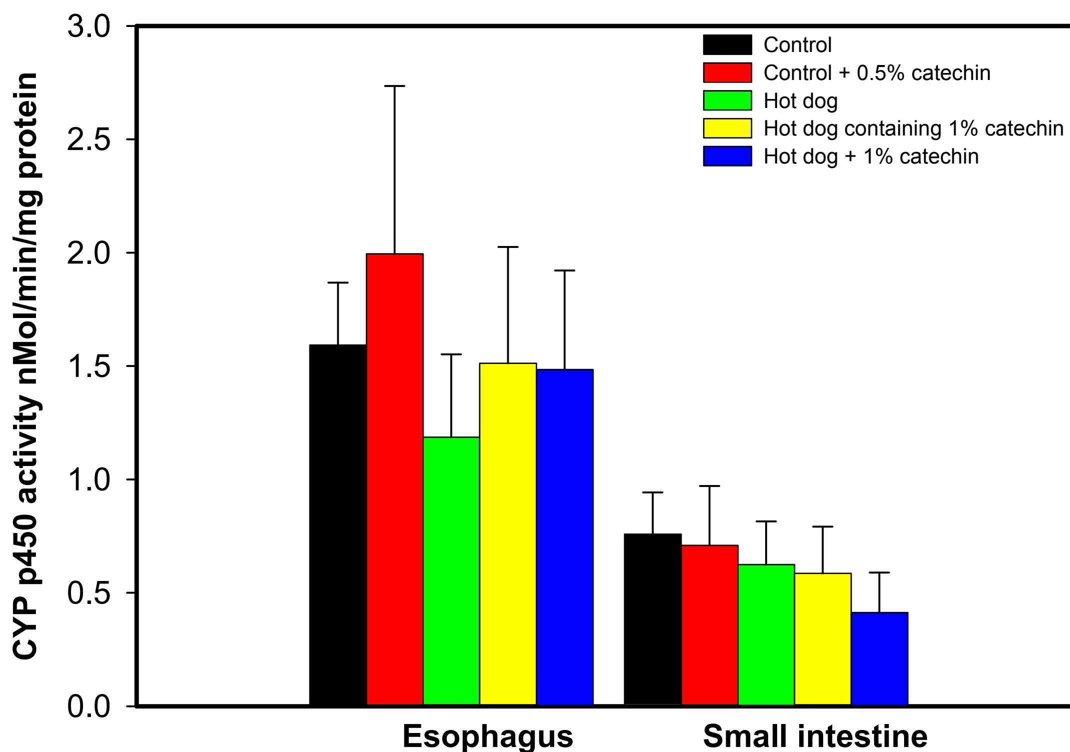

Figure 26: CYP1A1/A2 activity in esophagus and small intestine microsomes as determined by EROD assay. Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); HD (hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients); or PH (hot dog fed with 1:1 supplemented nutrients containing 1% green tea catechins). Diets were fed for 21 days (Mean±SEM; n=6).

The esophagus expressed CYP1A1 and 1A2 therefore EROD activity level measured for esophagus measures both types of CYP(45). Small intestine on the other hand expresses only CYP1A1 so the level of activity measured by the EROD assay reflects the level of CYP1A1 and not CYP1A2 (45). Figure 26 illustrates the change in CYP1A1/A2 activity in rat esophagus and small intestinal microsomes. There were no significant changes in CYP1A1/A2 in either esophagus or small intestinal microsomes.

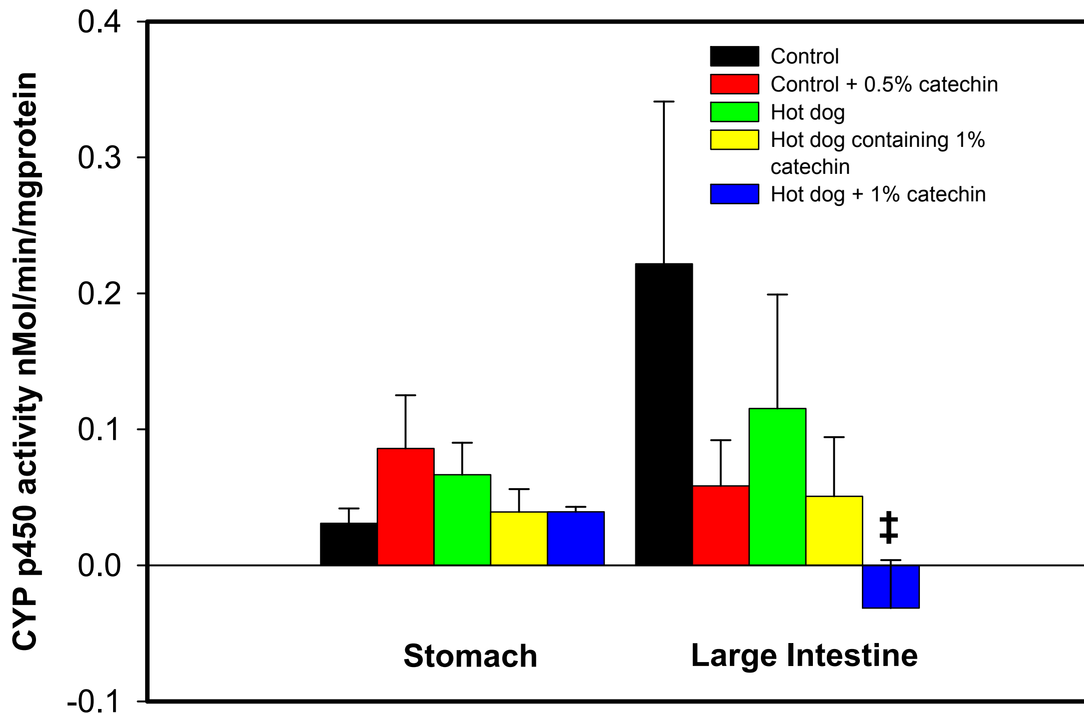

Figure 27: CYP1A1/A2 activity in stomach and large intestine microsomes as determined by EROD assay. Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); HD (hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients); or PH (hot dog fed with 1:1 supplemented nutrients containing 1% green tea catechins). Diets were fed for 21 days (Mean±SEM; n=6).

‡ indicates a significant hot dog/catechin effect ($p=0.019$)

Figure 27 illustrates the results from the EROD assay conducted on stomach and large intestinal microsomes. Stomach and large intestinal mucosa express both CYP1A1 and CYP1A2(45). CYP1A1/A2 activity was significantly reduced in rats fed the PH diet when compared to the CON fed rats (p=0.019). The remaining diets had no effect on large intestinal microsomal CYP1A1/A2 activity. The dietary treatments had no significant effect on the stomach microsomal CYP1A1/A2 activity.

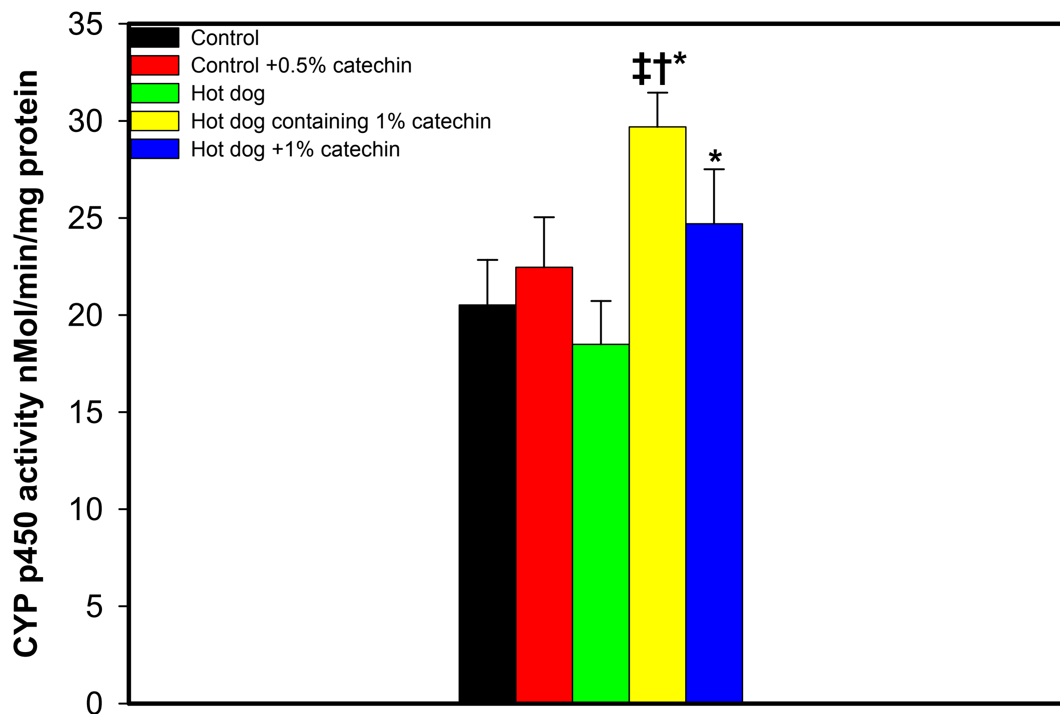

Figure 28: CYP1A1/A2 activity in liver microsomes as determined by EROD assay. Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); HD (hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients); or PH (hot dog fed with 1:1 supplemented nutrients containing 1% green tea catechins). Diets were fed for 21 days (Mean±SEM; n=6).

‡ indicates a significant hot dog/catechin effect (p=0.011); † indicates a significant hot dog effect (p=0.040); * indicates a significant catechin effect (p=0.003); * indicates a trend in catechin effect (p=0.075)

Figure 28 illustrates the effect of dietary treatments on the level of CYP1A2 activity in liver microsomes. Liver contains only CYP1A2, consequently the level of CYP activity measured by the EROD assay reflects only the level of CYP1A2(42) Rats fed HDGT diet had a significantly higher level CYP1A2 activity when compared to rats fed CON (p=0.011), CGT (p=0.04) or HD (p=0.003) diets. In addition, rats fed the PH diet did have a higher level of CYP1A2 activity in liver microsomes when compared to HD fed rats (p=0.075), the result was not significant but it could be considered a trend. The remaining treatments had no effect on the level of CYP activity measured by the EROD assay.

To summarize the western blot data, HD and CGT fed rats had a significantly lower amount of CYP2E1 in esophagus microsomes when compared to CON fed rats. In western blots of stomach microsomes CGT and PH fed rats had a significantly higher amount of CYP1A1/A2 when compared to CON fed rats. In western blots of liver microsomes HD fed rats had a significantly higher level of CYP1A1 when compared to CON fed rats. PH fed rats had a significantly higher level of CYP2E1 when compared to CON fed rats in western blots of liver microsomes. AhR was significantly higher in liver microsomes of HDGT fed rats when compared to CON fed rats. AhR was significantly higher in small intestinal microsomes of HD and HDGT fed rats when compared to CON fed rats. In the large intestine microsomes CGT and PH fed rats had significantly higher levels of CYP2E1 when compared to the CON fed rats.

To summarize the EROD assay results we see that the dietary treatment had no effect on the levels of CYP activity in the esophagus, stomach or small intestine. The large intestine of rats fed the PH diet had a significantly higher level of CYP1A1/A2 when compared to CON fed rats. Not surprisingly the liver showed the most significant dietary effects. The level of CYP1A2 activity in liver microsomes was significantly higher in rats fed HDGT diet when compared to CON, CGT, and HD fed rats. PH fed rats had a higher level of CYP activity in liver microsomes when compared to HD fed rats. The difference between CYP1A2 activity in PH fed rats when compared to HD fed rat was not significant but the p value could be considered a trend.

Results of phase 2 xenobiotic metabolizing enzymes

Phase 2 xenobiotic metabolizing enzymes conjugate bioactivated PAH, HCA and NA, usually making them more stable and water soluble so they can be excreted(42). The main phase 2 enzymes involved in safely conjugating bioactivated PAH, HCA and NA are glutathione S-transferase and UDP-glucosyl transferase (41).

In this experiment our goal was to examine the effect of 21 days of dietary treatment (outlined in the methods) on phase 2 xenobiotic enzyme activity. We hypothesize that the diets containing green tea should increase the activity of the GST and UGT families of phase 2 enzymes. Rats were fed their appointed diet starting at approximately 25 days of age and continued for 21 days. Rats grew

normally over the course of the experiment (figure 20). There was no statistical difference in the average body weights between the treatment groups ($p \geq 0.05$).

GST activity

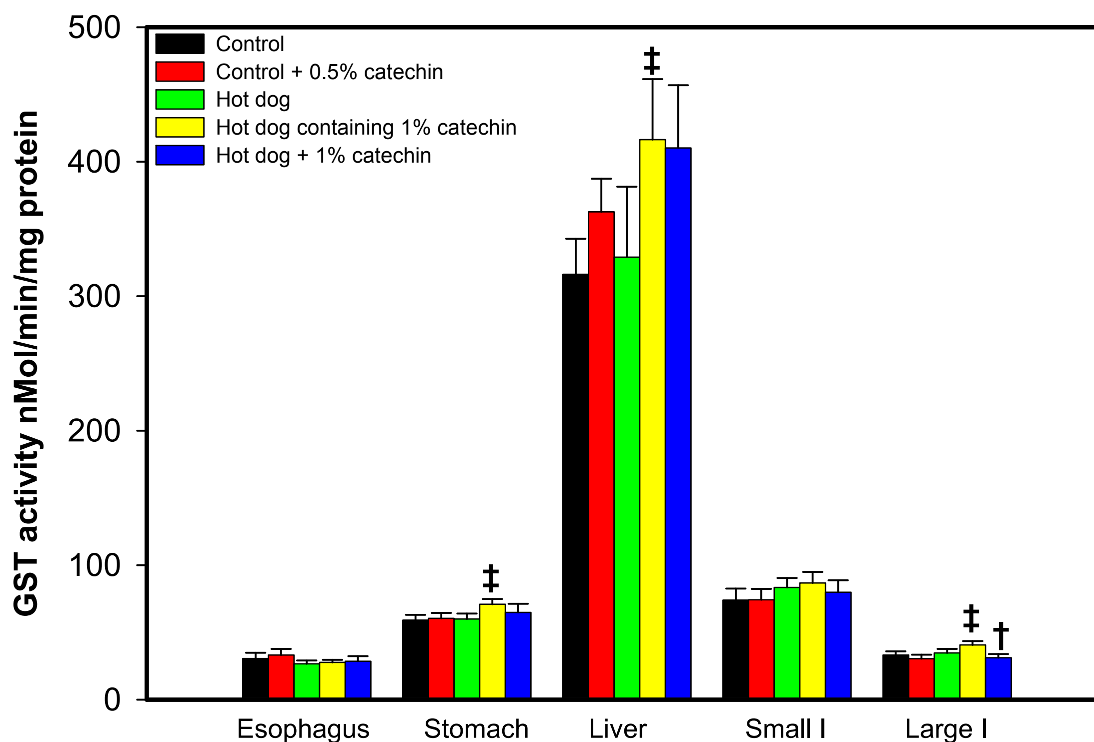

Figure 28: GST activity in gastrointestinal tissues and Liver cytosol (means±SEM; n=6). Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); HD (hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients); or PH (hot dog fed with 1:1 supplemented nutrients containing 1% green tea catechins). Diets were fed for 21 days. ‡ indicates a trend in hot dog/catechin effect (stomach, p=0.081; liver, p=0.094: Large intestine, p=0.074); † indicates a significant hot dog effect for CGT compared to HDGT (p=0.018) and HDGT compared to PH (p=0.027)

Figure 28 illustrates the activities of GST in the esophagus cytosol of rats fed each of the 5 dietary treatments outlined in the methods section. The dietary treatments had no significant effect on GST activity in the esophagus or stomach cytosol. In the liver we see elevated GST activity in HDGT and PH fed rats, suggesting a combined effect of meat and catechin intake. Although these changes are not significant the increase in GST activity in the liver cytosol of HDGT fed rats when compared to CON fed rats could be considered a trend (p=0.094). There was no significant difference between dietary treatments in the small intestine. The large intestine exhibits a significant increase in GST activity in HDGT fed rats when compared to CGT (p=0.018) or PH (p=0.027) fed rats. In addition, we see a non significant increase in GST activity in large intestinal cytosol of HDGT fed rats when compared to CON fed rats. Even though this increase is not significant it could be considered a trend towards significant (p=0.074).

UGT activity

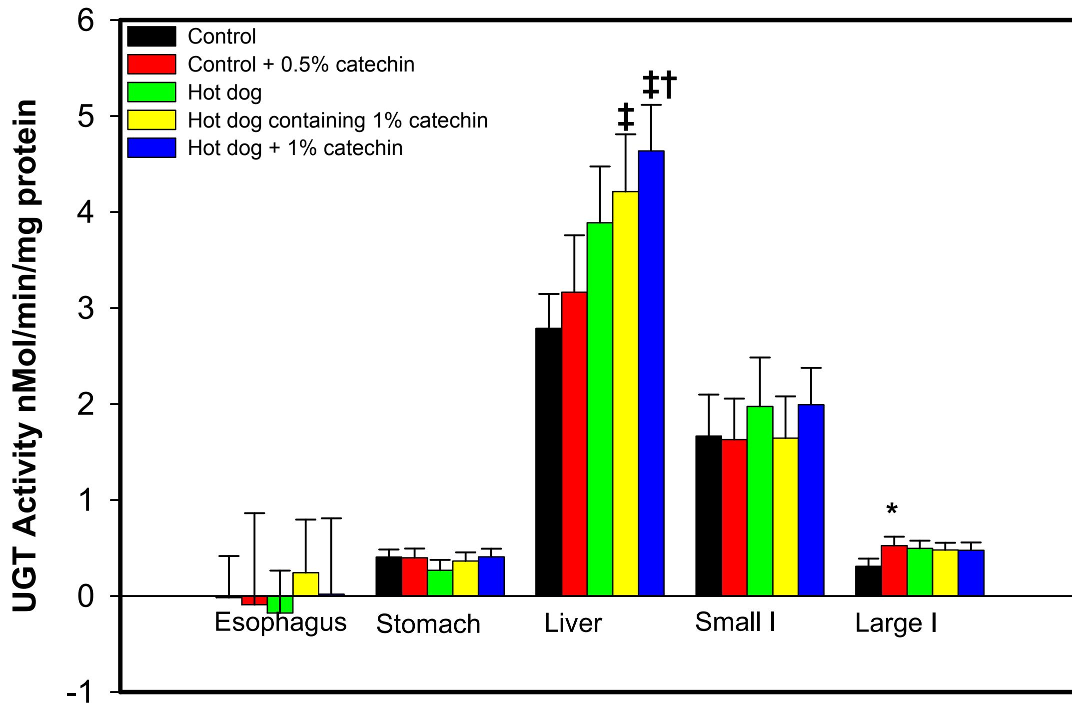

Figure 30: UGT activity in gastrointestinal tissues and liver microsomes (Mean ±SEM n=5 for esophagus and small intestine and n=6 for all other tissues). Diets: CON (a modified AIN 93g diet), CGT (control diet +0.5% green tea catechins); HD (hot dog diet fed 1:1 with supplemented nutrients); HDGT (hot dog containing 1% green tea catechins fed 1:1 with supplemented nutrients); or PH (hot dog fed with 1:1 supplemented nutrients containing 1% green tea catechins). Diets were fed for 21 days. ‡ indicates a trend in hot dog /catechin (p=0.070); ‡ indicates a significant hot dog/catechin effect (p=0.021); † indicates a trend in hot dog effect (p=0.061).

*** indicates a trend in catechin effect (p=0.076)**

Dietary treatments had no effect on UGT activity in esophagus or stomach microsomes (figure 30). Liver microsomes had a significant increase in UGT activity in PH fed rats when compared to CON (p=0.021). The level of UGT activity is higher but not significant in the liver microsomes of HDGT when compared to CON fed rats (p=0.070). PH fed rats had higher but not significant level of UGT activity when compared to CGT (p=0.061). There was no significant effect of diet on UGT activity in small intestine microsomes. UGT activity in large intestinal microsomes of CGT fed rats is higher, but not significantly so, when compared to the UGT level in CON fed rats (p=0.076).

To summarize the significant findings we see no significant dietary effect on the level of GST in esophagus and small intestine. In the stomach, HDGT fed rats did have a slight non significant elevation of GST activity when compared to CON. A similar non significant increase in GST is seen in liver of HDGT fed rats. In the large intestine there was significantly more GST in HDGT when compared to PH and CGT fed rats. A non significant increase in GST was seen in the large intestine of HDGT fed rats when compared to CON.

There were no significant dietary effects on UGT in the esophagus, stomach or the small intestine. In the large intestine HDGT fed rats had a significantly higher level of UGT activity when compared to CGT fed rats. CGT fed rats had a higher level of UGT activity in the large intestine when compared to CON fed rats however it was not significant. The liver was the organ that had the most dietary effects of all the tissues. PH fed rats had significantly higher UGT activity in the

liver when compared to CON fed rats. HDGT fed rats also had a higher level of UGT activity in the liver when compared to CON but the effect was not significant. PH fed rats also had a non significant increase in liver UGT activity when compared to CGT fed rats.

Discussion of phase 1 xenobiotic metabolism

This experiment was designed to test the hypothesis that green tea catechins could reduce CYP induction caused by the bioactivation of PAH, HCA and NA contained in hot dog. This hypothesis was based on both epidemiological and experimental evidence suggesting that frequent consumption of green tea catechins reduces CYP bioactivation and enhances phase 2 conjugating enzymes and that frequent consumption of PAH, HCA and NA contained in cooked and cured meat will induce CYP1A1, CYP1A2 and CYP2E1 in gastrointestinal tissues.

Esophagus

In this experiment we found green tea catechins contained in the CGT diet did not reduce the level of CYP1A1/A2 present in the esophagus. The consumption of hot dog as 50% of the dry matter intake did not induce CYP1A1/A2 in rats. The consumption of hot dog containing green tea had no significant effect on the level of CYP1A1/A2. Also, the feeding of green tea catechins together with hot dog did not have any significant effect on CYP1A1/A2 levels. The consumption of hot dogs containing nitrite had no significant effect on the level of CYP2E1.

The green tea catechins either contained in or fed at the same time as hot dog had no effect on the level of CYP2E1. There was no significant difference in AhR levels between treatment groups.

The lack of significant effect of the diets on the level of CYP1A1/A2 and 2E1 in the esophagus is unexpected. There is little direct evidence examining the effect of consuming cooked and cured meat on the level of CYP1A1/A2 and 2E1 in the esophagus. However, research conducted by Uno et al (2008) found that oral dosing of BAP (100 mg/kg) was able to induce CYP1A1 and CYP1A2 mRNA to twice the level seen in the sham dosed rats(115). In this experiment BAP was delivered by itself and not in conjunction with a meal. When meat is consumed the BAP is mixed in with the meat. Any surface BAP is mixed during chewing. This mixing could prevent bioactivation of PAH, HCA and NA and induction of CYP1A1/A2 and CYP2E1. As a result, a model based on gavaging BAP, which bypasses the mouth and esophagus, may not be the most realistic model to use when investigating lifestyle related cancers.

Stomach

In the stomach the CGT diet tea induced CYP1A1/A2. CYP1A1 induction was also seen in PH fed rats when green tea was fed at the same time as hot dog. The increase in CYP in CGT fed rats is mirrored in the EROD assay results. However, the EROD assay did not produce a significant difference between CON and CGT fed rats. The EROD assay for PH fed rats did not show the same results as the western blots. In the EROD assay results we see CYP1A1/1A2

activity is reduced in PH fed rats when compared CON where as the western blots show a significant increase in CYP1A1/A2 over Con fed rats. On possible explanation for this result is that CYP1A1/A2 is being induced but its activity is not increasing because of the presence of the catechins being fed at the same time as hot dog. So the PAH, HCA and NA in the hot dog are inducing CYP but the catechins are preventing CYP activity through another mechanism other than AhR.

The CON, HD and HDGT had no significant effect on the stomach microsomal CYP1A1/A2 activity. There was no significant difference in CYP2E1or AhR levels in rat stomach microsomes for any of the dietary treatments. In the stomach of CGT and PH fed rats CYP1A1/A2 was induced in the presence of green tea catechins. Anger et al (2005) saw a similar induction of CYP1A1 in ex vivo liver microsomes when exposed to green tea at low concentrations. This induction of CYP1A1 was suppressed at higher concentrations of catechins(116). In addition the low pH of the stomach environment may change the conformation of the catechins thus reducing their ability to bind to AhR which in turn would affect its ability to inhibit CYP1A1 induction.

Another possibility is that the highly exocrine environment of the stomach is affecting the ability of the catechins to be fully absorbed and therefore we are not seeing the full dose of green tea on the cells. This may also be the reason that hot dog did not induce CYP1A1/A2 or CYP2E1. The hot dog and powdered supplement are purified and refined. Consequently the diet may not be spending much time in the stomach before moving on to the small intestine. Low retention

time of the stomach is possibly affecting the induction of CYP1A1/A2, 2E1 and AhR.

Liver

The liver was an unexpected target for dietary catechins, PAH, HCA and NA because of its distance from the gastrointestinal tract. The liver of HD fed rats had a significant decrease in CYP1A2. This decrease was also reflected in a reduction in EROD activity. This result is unexpected as the liver has the greater amount of CYP1A2 when compared to gastrointestinal tissues as a result it was expected that CYP1A2 would be induced in response to hot dog. When 125 mg/kg BAP was gavaged into mice CYP1A2 was increased in the liver (117). One possible reason why Uno et al. observed induction of CYP, and we did not in this experiment is BAP was given as a single bolus dose rather than a slower release that would be seen when BAP is released from food being digested and absorbed into the body from the stomach and small intestine. BAP is not the only chemical contained in cooked meat that is metabolized by CYP1A2. Hirose et al (1999) found that when 200 mg/kg of PHIP was injected i.p. into rats they found that CYP1A2 was undetectable in the liver. However, when given in conjunction with a synthetic antioxidant CYP1A2 was increased(118). This result could also explain why rats fed HDGT diet had a significantly higher level of CYP1A2 activity when compared to rats fed CON, CGT or HD diets. A similar non significant increase in CYP1A2 activity was seen in the rats fed catechins in conjunction with hot dog (PH). These increases in activity were mirrored in the

western blot of CYP1A2. Interestingly, we also see a significant increase in AhR in rats consuming HDGT diet. This may be further indication that green tea in hot dogs may not be inhibiting CYP1A2 or 2E1 as we had hypothesized. Alternatively we could be seeing an increase in AhR because CYP1A2 and CYP2E1 are being inhibited and thus the body is compensating by increasing the receptor responsible for CYP1A2 and CYP2E1 transcription.

Rats fed the PH diet had a significant increase in liver CYP2E1 when compared to CON (p=0.028) while remaining diets had no significant effect. The purpose of feeding green tea extract at the same time as hot dog was to determine whether the cooking of the hot dog containing green tea was having any effect on CYP1A1/A2 or CYP2E1. This result indicates that cooking the green tea in the presence of the hot dog may induce 2E1, and that the catechins may be destroyed or chemically changed during the making, storing and cooking of hot dogs.

Green tea does inhibit the endogenous formation of NDMA (91). Choi et al reports that the addition of 0.6g of tea in 100mL of water inhibits NDMA excretion in humans by 60% (91). CYP2E1 is responsible for metabolizing NDMA; as a result one could infer that if NDMA excretion is being reduced CYP2E1 must be also reduced. However, another possibility is that green tea is reducing NDMA excretion because CYP2E1 is increasing and therefore more NDMA is being metabolized by 2E1. Unfortunately, Choi does not measure CYP2E1 activity or down stream metabolites of CYP2E1 mediated metabolism of NDMA.

Small intestine

Rats that consumed HD and HDGT diet had significantly higher levels of AhR in the small intestine when compared to CON fed rats. However this increase in AhR does not correspond to an increase in CYP1A1/1A2 levels. There were no significant changes in CYP1A1/A2 activity small intestinal microsomes. This is an unexpected result as other research has reported an increase in CYP1A1/1A2 mRNA in the small intestine of mice gavaged with BAP(115). Presumably the increase in mRNA should correspond to an increase in CYP1A1 and CYP1A2 protein. In addition, BAP orally administered to mice results in DNA adducts in the small intestine(37), which require bioactivation by CYP enzymes. CYP2E1 does increase in HD and HDGT fed rats in a similar pattern as seen in AhR. It is likely that the increase in AhR corresponds to the increase in CYP2E1. This indicates some level of NDMA bioactivation in response to feeding hot dog but not at significant levels. All diets in this experiment contained sufficient levels of vitamin C and vitamin E. Both of these vitamins are well accepted as antioxidants. Vermeer et al (1999) found that ingestion of ascorbic acid in conjunction with green tea resulted in a significant reduction in NDMA production in humans(119). In addition, it is also been shown that vitamin C (120) and green tea (121) consumed individually can reduce the level of NDMA production. The inclusion of vitamins C and E contained in all diets as well as green tea catechins contained in the CGT, HDGT and PH diets may have influenced the amount of NDMA formed.

Large intestine

Green tea has a significant effect on CYP2E1 activity in the large intestine. CYP2E1 was significantly increased in both CGT and PH fed rats when compared to CON. This result is interesting since green tea catechins have low bioavailability(77) and a half-life of 165min in rats(97). However with repeated exposure of to 0.6% green tea catechins in drinking water over 28 days, plasma levels of EGCG in rats peak at 15 days and continue at that level past 30 days of treatment indicating that a prolonged treatment appears to provide the greatest effect(97). Green tea catechin is proposed to inhibit AhR which we would expect to result in a decrease in 2E1(48;82). In rats, the proximal small intestine has the highest level of catechins when compared to plasma, kidney, liver and lung(122). However, EGCG has been shown to inhibit Pgp thus preventing the efflux of EGCG from the cell into the lumen of the intestine. Pgp is an ABC transporter present in large quantities on the apical surface of the small and large intestine.(76;123-125). Thus, the low bioavailability of EGCG and other catechins as assessed by blood measurements is not an accurate indicator of the levels that accumulate in gastrointestinal tissues. Approximately 40% of the catechins absorbed in the small intestine return to the intestine through bile as glucuronide or sulfate conjugates of methoxy-(+)-catechin. They are then deconjugated by large intestine bacteria back to catechins as well as phenolic acid and lactone. Approximately 60% of this metabolized catechin is reabsorbed by the large intestine via enterohepatic recycling(126-128). This coupled with the inhibition of

Pgp by ECGC may be the mechanism behind the proposed ability of green tea to prevent colon cancer.

EROD activity (normally attributed to CYP1A1/A2) was significantly reduced in rats fed the PH diet when compared to the CON fed rats. However the total amount of CYP1A1/A2 protein was not significantly reduced. Since the regulation of gene expression for CYP1A1/A2 and CYP2E1 significantly differ, and these isozymes metabolize different substrates it is quite possible that green tea will have a different effect on the toxicity of PAH and HCA vs. NA. In addition AhR levels were not affected by dietary treatments. Hosoya et al (2008) found that CYP1A1 can be induced in AhR deficient mice indicating that CYP1A1 can be induced by an AhR independent pathway(129). Thus it is not surprising that changing levels of CYP1A1 may not be reflected in AhR levels. Green tea catechins may inhibit CYP1A1 activity at high concentrations but activate CYP at low concentrations(116). To have some idea of the potential for affecting the expression and activity of various P450 isozymes more research will be required on the accumulation of catechins in colon tissue. This is complicated by variations in the food matrix (hot dog forming and cooking effects, vs. the usual exposure in teas), and also by differences in colon bacterial ecology between models.

In conclusion the changes are not all consistent, but they also aren’t very large in magnitude. However, the take home message from this experiment is that CYP1A1/A2 and CYP2E1 expression was very stable in response to a dramatic

cooked meat diet relative to experimental models with purified carcinogens(130;131) (132).

Discussion of phase 2 xenobiotic metabolizing enzymes

The purpose of this experiment was to examine the effect of feeding green tea catechins and cooked and cured meat on glutathione S-transferase (GST) and UDP-glucuronyl transferase (UGT) activities. Evidence from the literature suggests that green tea catechins should increase the level of cytosolic GST and microsomal UGT. Increase the levels of phase 2 xenobiotic metabolizing enzymes should increase the conjugation of bioactivated PAH, HCA and NA making them less reactive and more water soluble, allowing them to be safely excreted(42).

GST activity

Esophagus

GST levels in the esophagus did not change in response to either green tea or hot dog or both. The esophagus contains very low levels of GST in comparison to other gastrointestinal tissues. In addition little to no digestion of protein or fat takes place in the esophagus. Protein and fat are the carriers of HCA, or PAH as a result there would be little PAH or HCA available to absorb into esophageal mucosa. Therefore it is understandable that there would be little dietary effect on the esophagus. However, other research has shown that esophageal GST is

induced by other plant based polyphenols such as coumarin and α angelicalactone(133), so the results of the current study were somewhat unexpected.

Stomach

GST activity in rat stomach was not significantly affected by the consumption of green tea, hot dog or both together. This result is unexpected as other research conducted in rodent models has shown that the level of GST in the stomach is increased following dosing with a variety of compounds including coumarin and ellagic acid(134) and green tea polyphenols(133). In the HDGT fed rats we do see a non significant increase in GST in the stomach however in the CGT fed rats the level of GST was not different than control. It is possible that green tea requires the presence of PAH and HCA in order to increase GST.

Liver

In the liver rats fed CGT, HD, HDGT and PH fed rats all had non significant increases in GST level when compared to CON. Even though none of these increases are significant we can see a pattern that resembles the results seen in the stomach. GST is increased when green tea is given in conjunction with PAH and HCA. GST in the rat liver can be induced by green tea (135) and PAH ($\geq$10μM B[a]P) (136). Perhaps the consumption of the xenobiotics PAH and HCA

and green tea enhance the induction of GST more so than would be seen if each ingredient was fed individually.

Small intestine and Large intestine

Dietary treatments had no significant effect on GST activity level in small intestine however the large intestine appears to be more sensitive to green tea catechins. The lack of effect on small intestine is unexpected since GST levels are elevated in intestinal epithelial cell lines after repeated exposure to PHIP(137). In the large intestine GST activity is significantly increased in HDGT fed rats when compared to CGT or PH. This also follows the same pattern that was seen in the stomach and liver. Santana-Rios et al (2001) also found that white tea resulted in a significant in increase in colon GST(138). Unfortunately Santana-Rios did not measure GST in the animals that were dosed with tea and PHIP. In addition, a decrease in PHIP induced DNA adducts was observed in rats fed green tea catechins(89) thus giving further indication that green tea catechins may need PHIP or B[a]P in order to induce GST and therefore enhance glutathione conjugation to bioactivated HCA or PAH.

UGT activity

Esophagus and Stomach

There was no effect of dietary treatments on UGT activity in either esophagus or stomach. UGT activity is very low in the esophagus and stomach and food

spends very little time in the esophagus there for it is not surprising that there was no observed dietary effect.

Liver

UGT activity was significantly increased in rats fed PH when compared to CON. There was a non significant increase in UGT activity in HDGT fed rats when compared to CON fed rats. Exposure of hepatic cells to B[a]P in vitro induces UGT activity in the liver cell lines(139). In addition UGT liver increases by 50% when green tea is given as drinking water to rats(140). It appears that UGT levels are increased when green tea is given in conjunction with PHIP, HCA and NA. This supports the pattern we have seen in other tissues.

Small intestine and Large intestine

UGT activity in the small intestine was unaffected by dietary treatments. In the large intestine UGT activity displayed a non significant increase in CGT when compared to CON. This increase in UGT activity seen in CGT fed rats was also seen in mice dosed with EGCG (36). In rats the proximal small intestine has been found to have the highest level of catechins when compared to plasma, kidney, liver and lung(122). In addition this level of catechin is maintained longer in the small intestinal mucosa when compared to the plasma. This elevated level of catechin in the intestine may be responsible for some of the effects on UGT expression. In addition we also see a non significant increase in HD, HDGT and PH fed rats indicating that the consumption of cooked and cured meat in

conjunction with green tea did have an increased activity of UGT. This finding illustrates how when cooked and cured meat is fed in conjunction with green tea there is an enhanced level of UGT in the large intestine. The large intestine has higher levels of UGT and GST indicating that phase 2 enzymes may be more important in xenobiotic metabolism of PAH, HCA and NA.

Chapter 4: General discussion

The consumption of cooked and cured meats is associated via epidemiological evidence with an increased risk of developing gastrointestinal tract cancers(2). In addition, the consumption of phytochemicals in green tea has also been associated with a reduced risk of gastrointestinal tract cancers(2). However, the epidemiology in both of these areas remains somewhat controversial and, by its nature, does not prove that the relationships are causative.

Interactions between the families of chemicals contained in both green tea and cooked and cured meat is poorly understood. Bioactivation can occur in any cell that contains the appropriate CYP enzymes, in this case CYP1A1, 1A2 or 2E1. Once ingested, BaP, PhIP and DMN pass through the esophagus, stomach, small intestine and large intestine, making these tissues prime targets of bioactivation and conjugation. Chronic exposure to complex mixtures of secondary carcinogens and phytochemicals can significantly alter gene expression and change phase 1 and 2 xenobiotic metabolism.

In the long term study we set out to establish whether the consumption of cooked and cured meat causes gastrointestinal tumours in rats fed a physiologically relevant source of cooked and cured meat, albeit in rather large quantities. In addition we created a functional food combining the protective effects of green tea with a potentially carcinogenic cooked and cured meat. During this long term trial we found that consumption of cooked and cured meat did not result in a significant increase in gastrointestinal tumours in rats. The consumption of green tea had no effect on either the incidence of gastrointestinal cancers or the survival of animals with cancer possibly because the concentration of EGCG in

plasma decreases with prolonged consumption resulting in negligible long term effects. The lifetime consumption of hot dog did not cause an increase in gastrointestinal cancers. In fact, animals consuming grilled hot dog as 50% of dietary dry matter had significantly fewer cancers when compared to the combined control group. In society we often see the consumption of high levels of cooked and cured meats pairing with other poor life style choices such as alcohol consumption, cigarette smoking, low fruit and vegetable intake, low whole grain intake and lack of vitamin and mineral supplementation. Consequently cancer incidence that is apparently associated with cooked and cured meat intake may also be influenced by other poor life style choices(2;141). Most epidemiological studies take great pains to control for these additional lifestyle factors however confounding factors such as dietary recall error are difficult to completely control. As a result the discrepancy between the expected outcome as dictated by epidemiological evidence and the actual outcome of this experiment could have been a result of our controlling for the confounding factors that often plague epidemiological research.

The lifetime consumption of hot dog containing green tea had no effect on the incidence of gastrointestinal cancer; however it did result in a significantly lower incidence of one specific cancer, that being LGL leukemia. This effect could also be the result of a hormetic response to toxic components of the meat, or the result of green tea catechins. Green tea catechins may be more effective when paired with a source of xenobiotic stress such as PHIP, BaP or DMA present in hot dog and hot dog containing green tea. Hormesis is characterized by a “J”

shaped dose response curve. When hormesis is taking effect the drug or in this case food causes a toxic response at relatively low doses but as the dose increases the response of the organism decreases.

The theory of hormesis is supported by an early pilot project conducted by our lab (unpublished results shown in Appendix 4). Young, male, F344 rats were fed either grilled (HD) (a source of HCA and NA) or non-grilled (mainly NA) hot dog (ngHD) for 16 days followed by 1 day of control feeding. Following this protocol the rats were immediately challenged with 30 mg/kg of ethylnitrosourea (ENU). DNA damage in the bone marrow was then measured using the micronucleus assay. The hot dog feeding conferred a significant degree of protection against the genotoxic damage caused by ENU. This indicates that HCA ingested over 16 days may play a major role in hormesis and thereby providing some protection against genotoxic insult. In addition the ngHD diet also caused a significant decrease in DNA damage caused by diet indicating that nitrosamines may also play a role in providing DNA protection. In addition, further investigation conducted by our lab into the hormesis theory examined the effect of single bout feeding of hot dog on the incidence of DNA damage. DNA damage was assessed using the micronucleus assay. In this experiment either hot dog (HD) or non grilled hot dog (ngHD) was fed for 48 hours. A non significant increase in DNA damage was seen in the hot dog group indicating the early stages of the classic “J” shaped dose response curve seen during hormetic responses. Interestingly, in this experiment the ngHD diet caused a non significant decrease in DNA damage. This result indicates that HCA may play a

larger role in the hormetic response than nitrosamines (unpublished results shown in Appendix 4).

The theory of hormesis poses some interesting questions about the possible mechanisms responsible for the protective effects seen in the results from this experiment. Future studies should investigate the effect of consumption of hot dog and hot dog containing green tea on the level of DNA repair enzymes such as methyl transferase this will help determine whether hormesis is responsible for the protective effect on the hot dog and hot dog containing green tea. In addition a time course of hot dog consumption could help to elucidate the level of hot dog required to see the beneficial effect.

Consumption of cooked and cured meat does not have any effect on induction of CYP1A1/A2 or CYP2E1 in the esophagus. In addition there was no effect of cooked and cured meat or green tea catechin consumption on the level of GST or UGT in the esophagus. When food is consumed the pro-carcinogens are contained within the food and on the surface. Any PAH, HCA, NA will be mixed during chewing. In addition there is little digestive action that takes place in the mouth or esophagus so HCA contained in the food will not be exposed to the mucosal lining of the esophagus. Most of the risk of developing esophageal cancer is associated with the exposure of the esophagus to very hot beverages, cigarette smoke or heavily salt cured fish. All of these items contain readily available pro-carcinogens that can be absorbed across the mucosal lining of the

esophagus. Often these risk factors are performed in conjunction with cooked and cured meat thus it is often difficult to separate the multiple risk factors.

In the stomach the main effect seems to be from green tea. The consumption of green tea catechins resulted in a significant increase in CYP1A1/A2 in the stomach. In addition we see a similar induction of CYP1A1/A2 in the stomach of rats fed hot dog and green tea. However there was no dietary effect on CYP2E1, UGT or GST. In the stomach green tea does not appear to be inducing phase 2 enzymes and it appears to be inducing phase 1 enzymes. This is the opposite of what has been reported in the literature. It is possible that the low pH of the stomach may have altered the catechins ability to bind to AhR. However we would expect to see increased levels of AhR in the stomach mucosal lining if CYP1A1/A2 was being induced. In addition, in the stomach we see the beginning of enzymatic proteolysis of hot dog protein by pepsin. When green tea catechins are mixed with protein; pepsin may alter the ability of catechins to block CYP1A1/A2 induction. An altered form of EGCG could explain why there is no upregulation of GST and UGT when CYP is induced. Future experiments should examine other nuclear receptors to determine whether CYP, UGT or GST upregulation is being controlled by another receptor. In addition, future experiments could examine the effect of protein and pepsin on EGCG function.
In the liver we see a reduction of CYP1A2 activity with the feeding of hot dog but an increase in CYP1A2 activity in HDGT fed rats. Because of the high level of CYP1A2 and GST in the liver it is somewhat surprising to see no significant

increase in GST. However UGT was significantly increased in the liver of rats fed green tea catechins at the same time as hot dog. This would indicate that green tea fed at the same time as hot dog results in induction of UGT and thus enhancing conjugation of bioactivated NDMA. The liver is one step removed from the absorption of PAH, HCA and NA. CYP1A1/A2 in the stomach and small intestine will be the first to bioactivate HCA, PAH and NA. So by the time the liver receives any PAH, HCA or NA it is at a lower quantity than the level seen by the CYP, GST and UGT in the stomach and small intestinal mucosa.

In the small intestine we see hot dog increasing the level of AhR. In addition we see an increase in CYP2E1 but not CYP1A1/A2 in response to meat intake. CYP2E1 is induced by DMN while CYP1A1/A2 is induced by PAH and HCA(26;57). This indicates that nitrosamine bioactivated to DMN may play a bigger role than bioactivated HCA and PAH possible because DMN is formed endogenously which may lead to larger quantities when compared to PAH and HCA. The level of PAH and HCA may be declining as the food item progresses down the gastrointestinal tract. Not surprisingly we do not see an increase in GST. If CYP1A1/A2 is not being induced and consequently there is little bioactivation of PAH, and HCA it is reasonable to expect that GST would not be induced. However, with the increase in CYP2E1 it is expected that UGT would be increased as well. This was not the case. UGT did not increase in response to increased levels of NDMA. Future research needs to examine the level of endogenous nitrosamine production at the different stages of the small intestine to confirm that nitrosamine is being produced in the stomach and along the small

intestine. Future research should also look at microsomes of the entire small intestine and microsomes and cytosol of the duodenum, jejunum and ileum need to be examined for level of CYP, GST and UGT activity to determine which location along the small intestine plays a role in UGT, GST and CYP concentrations.

In the large intestine green tea catechins fed with hot dog significantly increased the level of CYP2E1 while CYP1A1/A2 was not affected. However it is green tea catechins fed alone that significantly increases the level of UGT in the large intestine. As food passes down the gastrointestinal tract PAH, HCA and NA are absorbed from the food, bioactivated and conjugated by the upper intestinal tract. Some of the conjugates may be excreted in bile which then reaches the large intestine. We do not know how much microbial populations play a role in metabolizing PAH, HCA and NA. There should be little of these xenobiotics available for bioactivation in the large intestine. Therefore the large intestinal phase 1 and phase 2 enzymes may play more of a role in metabolizing the products of microbial xenobiotic metabolism. Future experiments should aim to determine the effect of microbial bioactivation and conjugating enzymes.

In this experiment we examined the effect of cooked and cured meat consumption on the level of xenobiotic metabolism in the gastrointestinal tissues and liver. Contrary to what we expected, the consumption of hot dog with no green tea did not have an effect on the xenobiotic metabolizing enzymes of the gastrointestinal tract and it may have played a role in lowering phase 1 bioactivation of PAH, HCA and NA in the liver. When green tea and hot dog

were fed together or when hot dog containing green tea was consumed the different tissues of the gastrointestinal tract responded very individually. On the whole, changes that did occur were not large in magnitude. As a result we should consider that consumption of cooked and cured meat alone does not cause the upregulation of phase 1 or 2 enzymes unless green tea catechin is present.

Strengths and Limitations

The main strength of this project is that is addresses an area of research that has not been addressed to date. Previous rodent models of gastrointestinal tract cancers outlined in the literature use a purified carcinogen to mimic dietary carcinogens and to induce gastrointestinal cancers. This is an effective model for examining the relationship between a single injected or orally dosed carcinogen on the incidence of gastrointestinal cancer. However this model fails to examine how this carcinogen behaves in the presence of food in the gastrointestinal tract. Our model takes the first step in examining the effect of dietary carcinogen fed as part of the diet without the use of further purified carcinogens. Further research is required to refine this model for future use. One of the limitations to our study was that in the first long term morbidity study we chose not to use equal sample sizes. The sample sizes were chosen with the literature supported, preconceived notion that animals consuming cooked and cured meat over a lifetime would succumb to gastrointestinal cancer before

animals fed a control diet. Unfortunately the smaller sample size in the control groups may have had an effect on statistical analysis of this study resulting in a lack of significant effect on some of the treatments.

Reference List

1. Truswell AS. Meat consumption and cancer of the large bowel. Eur.J.Clin.Nutr. 2002;56 Suppl 1:S19-S24.

2. World Cancer Research Fund/ American Institute for Cancer Research. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. Washington DC: AICR, 2007.

3. Huxley RR, Ansary-Moghaddam A, Clifton P, Czernichow S, Parr CL, Woodward M. The impact of dietary and lifestyle risk factors on risk of colorectal cancer: a quantitative overview of the epidemiological evidence. Int.J.Cancer 2009;125:171-80.

4. Knekt P, Steineck G, Jarvinen R, Hakulinen T, Aromaa A. Intake of fried meat and risk of cancer: a follow-up study in Finland. Int.J.Cancer 1994;59:756-60.

5. Iscovich JM, L'Abbe KA, Castelleto R et al. Colon cancer in Argentina. I: Risk from intake of dietary items. Int.J.Cancer 1992;51:851-7.

6. Sinha R, Chow WH, Kulldorff M et al. Well-done, grilled red meat increases the risk of colorectal adenomas. Cancer Res. 1999;59:4320-4.

7. Matos E, Brandani A. Review on meat consumption and cancer in South America. Mutat.Res. 2002;506-507:243-9.

8. Gunter MJ, Probst-Hensch NM, Cortessis VK, Kulldorff M, Haile RW, Sinha R. Meat intake, cooking-related mutagens and risk of colorectal adenoma in a sigmoidoscopy-based case-control study. Carcinogenesis 2005;26:637-42.

9. Sinha R, Peters U, Cross AJ et al. Meat, meat cooking methods and preservation, and risk for colorectal adenoma. Cancer Res. 2005;65:8034-41.

10. Shin A, Shrubsole MJ, Ness RM et al. Meat and meat-mutagen intake, doneness preference and the risk of colorectal polyps: the Tennessee Colorectal Polyp Study. Int.J.Cancer 2007;121:136-42.

11. Ferrucci LM, Sinha R, Graubard BI et al. Dietary meat intake in relation to colorectal adenoma in asymptomatic women. Am.J.Gastroenterol. 2009;104:1231-40.

12. Santarelli RL, Pierre F, Corpet DE. Processed meat and colorectal cancer: a review of epidemiologic and experimental evidence. Nutr.Cancer 2008;60:131-44.

13. Aune D, De Stefani E, Ronco A et al. Meat consumption and cancer risk: a case-control study in Uruguay. Asian Pac.J.Cancer Prev. 2009;10:429-36.

14. Norat T, Riboli E. Meat consumption and colorectal cancer: a review of epidemiologic evidence. Nutr.Rev. 2001;59:37-47.

15. Norat T, Lukanova A, Ferrari P, Riboli E. Meat consumption and colorectal cancer risk: dose-response meta-analysis of epidemiological studies. Int.J.Cancer 2002;98:241-56.

16. Narisawa T, Magadia NE, Weisburger JH, Wynder EL. Promoting effect of bile acids on colon carcinogenesis after intrarectal instillation of N-methyl-N'-nitro-N-nitrosoguanidine in rats. J.Natl.Cancer Inst. 1974;53:1093-7.

17. Chomchai C, Bhadrachari N, Nigro ND. The effect of bile on the induction of experimental intestinal tumors in rats. Dis.Colon Rectum 1974;17:310-2.

18. Babbs CF. Free radicals and the etiology of colon cancer. Free Radic.Biol.Med. 1990;8:191-200.

19. Mirvish SS, Haorah J, Zhou L, Clapper ML, Harrison KL, Povey AC. Total N-nitroso compounds and their precursors in hot dogs and in the gastrointestinal tract and feces of rats and mice: possible etiologic agents for colon cancer. J.Nutr. 2002;132:3526S-9S.

20. Knize MG, Salmon CP, Pais P, Felton JS. Food heating and the formation of heterocyclic aromatic amine and polycyclic aromatic hydrocarbon mutagens/carcinogens. Adv.Exp.Med.Biol. 1999;459:179-93.

21. Alaejos MS, Gonzalez V, Afonso AM. Exposure to heterocyclic aromatic amines from the consumption of cooked red meat and its effect on human cancer risk: a review. Food Addit.Contam Part A Chem.Anal.Control Expo.Risk Assess. 2008;25:2-24.

22. Jagerstad M, Skog K. Genotoxicity of heat-processed foods. Mutat.Res. 2005;574:156-72.

23. Dietrich CG, de Waart DR, Ottenhoff R, Schoots IG, Elferink RP. Increased bioavailability of the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in MRP2-deficient rats. Mol.Pharmacol. 2001;59:974-80.

24. Dingley KH, Curtis KD, Nowell S, Felton JS, Lang NP, Turteltaub KW. DNA and protein adduct formation in the colon and blood of humans after exposure to a dietary-relevant dose of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Cancer Epidemiol.Biomarkers Prev. 1999;8:507-12.

25. Turesky RJ. Heterocyclic aromatic amine metabolism, DNA adduct formation, mutagenesis, and carcinogenesis. Drug Metab Rev. 2002;34:625-50.

26. Baranczewski P, Gustafsson JA, Moller L. DNA adduct formation of 14 heterocyclic aromatic amines in mouse tissue after oral administration and characterization of the DNA adduct formed by 2-amino-9H-pyrido[2,3-b]indole (AalphaC), analysed by 32P_HPLC. Biomarkers 2004;9:243-57.

27. Kakiuchi H, Watanabe M, Ushijima T et al. Specific 5'-GGGA-3'-->5'-GGA-3' mutation of the Apc gene in rat colon tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Proc.Natl.Acad.Sci.U.S.A 1995;92:910-4.

28. Ochiai M, Imai H, Sugimura T, Nagao M, Nakagama H. Induction of intestinal tumors and lymphomas in C57BL/6N mice by a food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Jpn.J.Cancer Res. 2002;93:478-83.

29. Kato T, Ohgaki H, Hasegawa H, Sato S, Takayama S, Sugimura T. Carcinogenicity in rats of a mutagenic compound, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline. Carcinogenesis 1988;9:71-3.

30. Ohgaki H, Hasegawa H, Suenaga M, Sato S, Takayama S, Sugimura T. Carcinogenicity in mice of a mutagenic compound, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) from cooked foods. Carcinogenesis 1987;8:665-8.

31. Ohgaki H, Kusama K, Matsukura N et al. Carcinogenicity in mice of a mutagenic compound, 2-amino-3-methylimidazo[4,5-f]quinoline, from broiled sardine, cooked beef and beef extract. Carcinogenesis 1984;5:921-4.

32. Simko P. Factors affecting elimination of polycyclic aromatic hydrocarbons from smoked meat foods and liquid smoke flavorings. Mol.Nutr.Food Res. 2005;49:637-47.

33. Phillips DH. Polycyclic aromatic hydrocarbons in the diet. Mutat.Res. 1999;443:139-47.

34. Ramesh A, Walker SA, Hood DB, Guillen MD, Schneider K, Weyand EH. Bioavailability and risk assessment of orally ingested polycyclic aromatic hydrocarbons. Int.J.Toxicol. 2004;23:301-33.

35. Laurent C, Feidt C, Lichtfouse E, Grova N, Laurent F, Rychen G. Milk--blood transfer of (14)C-tagged polycyclic aromatic hydrocarbons (PAHs) in pigs. J.Agric.Food Chem. 2001;49:2493-6.

36. Zhang ZM, Yang XY, Yuan JH, Sun ZY, Li YQ. Modulation of NRF2 and UGT1A expression by epigallocatechin-3-gallate in colon cancer cells and BALB/c mice. Chin Med.J.(Engl.) 2009;122:1660-5.

37. Helleberg H, Xu H, Ehrenberg L, Hemminki K, Rannug U, Tornqvist M. Studies of dose distribution, premutagenic events and mutation frequencies for benzo[a]pyrene aiming at low dose cancer risk estimation. Mutagenesis 2001;16:333-7.

38. Gangolli SD, van den Brandt PA, Feron VJ et al. Nitrate, nitrite and N-nitroso compounds. Eur.J.Pharmacol. 1994;292:1-38.

39. Luca D, Raileanu L, Luca V, Duda R. Chromosomal aberrations and micronuclei induced in rat and mouse bone marrow cells by sodium nitrate. Mutat.Res. 1985;155:121-5.

40. van Logten MJ, den Tonkelaar EM, Kroes R, Berkvens JM, van Esch GJ. Long-term experiment with canned meat treated with sodium nitrite and glucono-delta-lactone in rats. Food Cosmet.Toxicol. 1972;10:475-88.

41. Jospehy D. Molecular Toxicology. New York: Oxford University Press, 1997.

42. Hodgson EaGJ. Metabolism of toxicants: phase I reactions and pharmacogenetics. In: Hodgson EaSR, ed. Introduction to biochemical toxicology. New York: Wiley 2001:67-113.

43. Kong AN, Owuor E, Yu R et al. Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). Drug Metab Rev. 2001;33:255-71.

44. Anzenbacher P, Anzenbacherova E. Cytochromes P450 and metabolism of xenobiotics. Cell Mol.Life Sci. 2001;58:737-47.

45. Ding X, Kaminsky LS. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. Annu.Rev.Pharmacol.Toxicol. 2003;43:149-73.

46. Tirona RG, Kim RB. Nuclear receptors and drug disposition gene regulation. J.Pharm.Sci. 2005;94:1169-86.

47. Palermo CM, Hernando JI, Dertinger SD, Kende AS, Gasiewicz TA. Identification of potential aryl hydrocarbon receptor antagonists in green tea. Chem.Res.Toxicol. 2003;16:865-72.

48. Yin Z, Henry EC, Gasiewicz TA. (-)-Epigallocatechin-3-gallate is a novel Hsp90 inhibitor. Biochemistry 2009;48:336-45.

49. Rushmore TH, Kong AN. Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes. Curr.Drug Metab 2002;3:481-90.

50. di Masi A, Marinis ED, Ascenzi P, Marino M. Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. Mol.Aspects Med. 2009;30:297-343.

51. Kwak MK, Wakabayashi N, Kensler TW. Chemoprevention through the Keap1-Nrf2 signaling pathway by phase 2 enzyme inducers. Mutat.Res. 2004;555:133-48.

52. Na HK, Surh YJ. Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG. Food Chem.Toxicol. 2008;46:1271-8.

53. Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. Annu.Rev.Pharmacol.Toxicol. 2005;45:51-88.

54. Turesky RJ. Interspecies metabolism of heterocyclic aromatic amines and the uncertainties in extrapolation of animal toxicity data for human risk assessment. Mol.Nutr.Food Res. 2005;49:101-17.

55. Kim D, Guengerich FP. Cytochrome P450 activation of arylamines and heterocyclic amines. Annu.Rev.Pharmacol.Toxicol. 2005;45:27-49.

56. Nowell SA, Ahn J, Ambrosone CB. Gene-nutrient interactions in cancer etiology. Nutr.Rev. 2004;62:427-38.

57. Boelsterli UA. Mechanistic toxicology. London: Taylor Francis Publishers, 2003.

58. Culp SJ, Gaylor DW, Sheldon WG, Goldstein LS, Beland FA. A comparison of the tumors induced by coal tar and benzo[a]pyrene in a 2-year bioassay. Carcinogenesis 1998;19:117-24.

59. Brooks RA, Gooderham NJ, Edwards RJ, Boobis AR, Winton DJ. The mutagenicity of benzo[a]pyrene in mouse small intestine. Carcinogenesis 1999;20:109-14.

60. Lijinsky W. N-Nitroso compounds in the diet. Mutat.Res. 1999;443:129-38.

61. Sheweita SA. Drug-metabolizing enzymes: mechanisms and functions. Curr.Drug Metab 2000;1:107-32.

62. Weisburger JH. Worldwide prevention of cancer and other chronic diseases based on knowledge of mechanisms. Mutat.Res. 1998;402:331-7.

63. Berlau J, Glei M, Pool-Zobel BL. Colon cancer risk factors from nutrition. Anal.Bioanal.Chem. 2004;378:737-43.

64. Levi F, Pasche C, Lucchini F, Bosetti C, La Vecchia C. Processed meat and the risk of selected digestive tract and laryngeal neoplasms in Switzerland. Ann.Oncol. 2004;15:346-9.

65. Lunet N, Lacerda-Vieira A, Barros H. Fruit and vegetables consumption and gastric cancer: a systematic review and meta-analysis of cohort studies. Nutr.Cancer 2005;53:1-10.

66. Lunet N, Valbuena C, Vieira AL et al. Fruit and vegetable consumption and gastric cancer by location and histological type: case-control and meta-analysis. Eur.J.Cancer Prev. 2007;16:312-27.

67. Larsson SC, Bergkvist L, Wolk A. Fruit and vegetable consumption and incidence of gastric cancer: a prospective study. Cancer Epidemiol.Biomarkers Prev. 2006;15:1998-2001.

68. Lai PK, Roy J. Antimicrobial and chemopreventive properties of herbs and spices. Curr.Med.Chem. 2004;11:1451-60.

69. Fujiki H, Suganuma M, Imai K, Nakachi K. Green tea: cancer preventive beverage and/or drug. Cancer Lett. 2002;188:9-13.

70. Fleischauer AT, Poole C, Arab L. Garlic consumption and cancer prevention: meta-analyses of colorectal and stomach cancers. Am.J.Clin.Nutr. 2000;72:1047-52.

71. Myung SK, Bae WK, Oh SM et al. Green tea consumption and risk of stomach cancer: a meta-analysis of epidemiologic studies. Int.J.Cancer 2009;124:670-7.

72. Sun CL, Yuan JM, Koh WP, Yu MC. Green tea, black tea and colorectal cancer risk: a meta-analysis of epidemiologic studies. Carcinogenesis 2006;27:1301-9.

73. Zhou Y, Li N, Zhuang W et al. Green tea and gastric cancer risk: meta-analysis of epidemiologic studies. Asia Pac.J.Clin.Nutr. 2008;17:159-65.

74. Hou Z, Lambert JD, Chin KV, Yang CS. Effects of tea polyphenols on signal transduction pathways related to cancer chemoprevention. Mutat.Res. 2004;555:3-19.

75. Saleem M, Adhami VM, Siddiqui IA, Mukhtar H. Tea beverage in chemoprevention of prostate cancer: a mini-review. Nutr.Cancer 2003;47:13-23.

76. Lambert JD, Yang CS. Cancer chemopreventive activity and bioavailability of tea and tea polyphenols. Mutat.Res. 2003;523-524:201-8.

77. Moyers SB, Kumar NB. Green tea polyphenols and cancer chemoprevention: multiple mechanisms and endpoints for phase II trials. Nutr.Rev. 2004;62:204-11.

78. Chow HH, Hakim IA, Vining DR et al. Effects of repeated green tea catechin administration on human cytochrome P450 activity. Cancer Epidemiol.Biomarkers Prev. 2006;15:2473-6.

79. Fujiki H, Suganuma M, Okabe S et al. Cancer inhibition by green tea. Mutat.Res. 1998;402:307-10.

80. ESTER IAKMIU CAMARGO Alissana (1) , ALESSANDRA ERDEI DAGUER Danielle (1) , SABBATINI BARBOSA Decio. Green tea exerts antioxidant action in vitro and its consumption increases total serum antioxidant potential in normal and dyslipidemic subjects. Nutrition research 2006;26:626.

81. Belguise K, Guo S, Yang S et al. Green tea polyphenols reverse cooperation between c-Rel and CK2 that induces the aryl hydrocarbon receptor, slug, and an invasive phenotype. Cancer Res. 2007;67:11742-50.

82. Palermo CM, Westlake CA, Gasiewicz TA. Epigallocatechin gallate inhibits aryl hydrocarbon receptor gene transcription through an indirect mechanism involving binding to a 90 kDa heat shock protein. Biochemistry 2005;44:5041-52.

83. Williams SN, Shih H, Guenette DK et al. Comparative studies on the effects of green tea extracts and individual tea catechins on human CYP1A gene expression. Chem.Biol.Interact. 2000;128:211-29.

84. Canadian Cancer Society steering committee. Canadian Cancer Statistics 2009. Toronto: Canadian Cancer Society, 2009.

85. Ferrucci LM, Cross AJ, Graubard BI et al. Intake of meat, meat mutagens, and iron and the risk of breast cancer in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. Br.J.Cancer 2009;101:178-84.

86. Gunter MJ, Probst-Hensch NM, Cortessis VK, Kulldorff M, Haile RW, Sinha R. Meat intake, cooking-related mutagens and risk of colorectal adenoma in a sigmoidoscopy-based case-control study. Carcinogenesis 2005;26:637-42.

87. Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. J.Nutr. 1997;127:838S-41S.

88. Caldwell DJ. Review of mononuclear cell leukemia in F-344 rat bioassays and its significance to human cancer risk: A case study using alkyl phthalates. Regul.Toxicol.Pharmacol. 1999;30:45-53.

89. Lin DX, Thompson PA, Teitel C, Chen JS, Kadlubar FF. Direct reduction of N-acetoxy-PhIP by tea polyphenols: a possible mechanism for chemoprevention against PhIP-DNA adduct formation. Mutat.Res. 2003;523-524:193-200.

90. Jiang T, Glickman BW, de Boer JG. Protective effect of green tea against benzo[a]pyrene-induced mutations in the liver of Big Blue transgenic mice. Mutat.Res. 2001;480-481:147-51.

91. Choi SY, Chung MJ, Sung NJ. Volatile N-nitrosamine inhibition after intake Korean green tea and Maesil (Prunus mume SIEB. et ZACC.) extracts with an amine-rich diet in subjects ingesting nitrate. Food Chem.Toxicol. 2002;40:949-57.

92. Patricia Lang and William White. Pathology of the aging rat. International Life Sciences, 1994.

93. McKiernan SH, Bua E, McGorray J, Aiken J. Early-onset calorie restriction conserves fiber number in aging rat skeletal muscle. FASEB J. 2004;18:580-1.

94. Chen YK, Lee CH, Wu IC et al. Food intake and the occurrence of squamous cell carcinoma in different sections of the esophagus in Taiwanese men. Nutrition 2009;25:753-61.

95. Shimizu M, Shirakami Y, Sakai H et al. (-)-Epigallocatechin gallate suppresses azoxymethane-induced colonic premalignant lesions in male C57BL/KsJ-db/db mice. Cancer Prev.Res.(Phila Pa) 2008;1:298-304.

96. Ui A, Kuriyama S, Kakizaki M et al. Green tea consumption and the risk of liver cancer in Japan: the Ohsaki Cohort study. Cancer Causes Control 2009.

97. Kim S, Lee MJ, Hong J et al. Plasma and tissue levels of tea catechins in rats and mice during chronic consumption of green tea polyphenols. Nutr.Cancer 2000;37:41-8.

98. Pierre F, Freeman A, Tache S, Van der MR, Corpet DE. Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons. J.Nutr. 2004;134:2711-6.

99. Pence BC, Landers M, Dunn DM, Shen CL, Miller MF. Feeding of a well-cooked beef diet containing a high heterocyclic amine content enhances

colon and stomach carcinogenesis in 1,2-dimethylhydrazine-treated rats. Nutr.Cancer 1998;30:220-6.

100. Hollman PC, Het Hof KH, Tijburg LB, Katan MB. Addition of milk does not affect the absorption of flavonols from tea in man. Free Radic.Res. 2001;34:297-300.

101. Ishmael J, Dugard PH. A review of perchloroethylene and rat mononuclear cell leukemia. Regul.Toxicol.Pharmacol. 2006;45:178-84.

102. Cao J, Ren LL, Liu JW, Li Y, Qu HY. Gene expression spectra in human leukemia HL-60 cells treated with EGCG. Mutat.Res. 2004;556:193-200.

103. Peters JM, Preston-Martin S, London SJ, Bowman JD, Buckley JD, Thomas DC. Processed meats and risk of childhood leukemia (California, USA). Cancer Causes Control 1994;5:195-202.

104. Sarasua S, Savitz DA. Cured and broiled meat consumption in relation to childhood cancer: Denver, Colorado (United States). Cancer Causes Control 1994;5:141-8.

105. Calabrese EJ, Baldwin LA. The frequency of U-shaped dose responses in the toxicological literature. Toxicol.Sci. 2001;62:330-8.

106. Maekawa A, Ogiu T, Onodera H et al. Carcinogenicity studies of sodium nitrite and sodium nitrate in F-344 rats. Food Chem.Toxicol. 1982;20:25-33.

107. Hord NG, Tang Y, Bryan NS. Food sources of nitrates and nitrites: the physiologic context for potential health benefits. Am.J.Clin.Nutr. 2009;90:1-10.

108. Gilchrist M, Winyard PG, Benjamin N. Dietary nitrate--good or bad? Nitric.Oxide. 2010;22:104-9.

109. Bryan NS, Calvert JW, Gundewar S, Lefer DJ. Dietary nitrite restores NO homeostasis and is cardioprotective in endothelial nitric oxide synthase-deficient mice. Free Radic.Biol.Med. 2008;45:468-74.

110. Wink DA, Vodovotz Y, Laval J, Laval F, Dewhirst MW, Mitchell JB. The multifaceted roles of nitric oxide in cancer. Carcinogenesis 1998;19:711-21.

111. Srivastava SK, Watkins SC, Schuetz E, Singh SV. Role of glutathione conjugate efflux in cellular protection against benzo[a]pyrene-7,8-diol-9,10-epoxide-induced DNA damage. Mol.Carcinog. 2002;33:156-62.

112. Patel HR, Hewer A, Phillips DH, Hayes JD, Wolf CR, Campbell FC. Metabolic competence and susceptibility of intestinal epithelium to genotoxic injury during regeneration. Carcinogenesis 1997;18:2171-7.

113. Kirkland JB. Phytochemicals, xenobiotic metabolism and carcinogenesis. In: Meckling KA, ed. Nutrient Drug Interactions. Boca Raton, FL.: Taylor and Francis, Inc. 2007.

114. Kong AN, Yu R, Chen C, Mandlekar S, Primiano T. Signal transduction events elicited by natural products: role of MAPK and caspase pathways in homeostatic response and induction of apoptosis. Arch.Pharm.Res. 2000;23:1-16.

115. Uno S, Dragin N, Miller ML, Dalton TP, Gonzalez FJ, Nebert DW. Basal and inducible CYP1 mRNA quantitation and protein localization throughout the mouse gastrointestinal tract. Free Radic.Biol.Med. 2008;44:570-83.

116. Anger DL, Petre MA, Crankshaw DJ. Heteroactivation of cytochrome P450 1A1 by teas and tea polyphenols. Br.J.Pharmacol. 2005;145:926-33.

117. Uno S, Dalton TP, Derkenne S et al. Oral exposure to benzo[a]pyrene in the mouse: detoxication by inducible cytochrome P450 is more important than metabolic activation. Mol.Pharmacol. 2004;65:1225-37.

118. Hirose M, Takahashi S, Ogawa K et al. Chemoprevention of heterocyclic amine-induced carcinogenesis by phenolic compounds in rats. Cancer Lett. 1999;143:173-8.

119. Vermeer IT, Moonen EJ, Dallinga JW, Kleinjans JC, van Maanen JM. Effect of ascorbic acid and green tea on endogenous formation of N-nitrosodimethylamine and N-nitrosopiperidine in humans. Mutat.Res. 1999;428:353-61.

120. Bartsch H, Ohshima H, Pignatelli B. Inhibitors of endogenous nitrosation. Mechanisms and implications in human cancer prevention. Mutat.Res. 1988;202:307-24.

121. Stich HF, Chan PK, Rosin MP. Inhibitory effects of phenolics, teas and saliva on the formation of mutagenic nitrosation products of salted fish. Int.J.Cancer 1982;30:719-24.

122. Chen L, Lee MJ, Li H, Yang CS. Absorption, distribution, elimination of tea polyphenols in rats. Drug Metab Dispos. 1997;25:1045-50.

123. Jodoin J, Demeule M, Beliveau R. Inhibition of the multidrug resistance P-glycoprotein activity by green tea polyphenols. Biochim.Biophys.Acta 2002;1542:149-59.

124. Raner GM, Cornelious S, Moulick K, Wang Y, Mortenson A, Cech NB. Effects of herbal products and their constituents on human cytochrome P450(2E1) activity. Food Chem.Toxicol. 2007;45:2359-65.

125. Zhou S, Lim LY, Chowbay B. Herbal modulation of P-glycoprotein. Drug Metab Rev. 2004;36:57-104.

126. Hackett AM, Griffiths LA. The disposition of 3-O-methyl-(+)-catechin in the rat and the marmoset following oral administration. Eur.J.Drug Metab Pharmacokinet. 1983;8:35-42.

127. Hollman PC, Tijburg LB, Yang CS. Bioavailability of flavonoids from tea. Crit Rev.Food Sci.Nutr. 1997;37:719-38.

128. Shaw IC, Griffiths LA. Identification of the major biliary metabolite of (+)-catechin in the rat. Xenobiotica 1980;10:905-11.

129. Hosoya T, Harada N, Mimura J et al. Inducibility of cytochrome P450 1A1 and chemical carcinogenesis by benzo[a]pyrene in AhR repressor-deficient mice. Biochem.Biophys.Res.Commun. 2008;365:562-7.

130. Mori Y, Niwa T, Toyoshi K et al. Carcinogenic activities of hydroxymethyl derivatives of 4-(dimethylamino)azobenzene in the liver of rats, mice, and hamsters. Exp.Pathol. 1984;26:15-9.

131. Mori Y, Koide A, Tatematsu K, Sugie S, Mori H. Effects of alpha-naphthyl isothiocyanate and a heterocyclic amine, PhIP, on cytochrome P-450, mutagenic activation of various carcinogens and glucuronidation in rat liver. Mutagenesis 2005;20:15-22.

132. Fontana RJ, Lown KS, Paine MF et al. Effects of a chargrilled meat diet on expression of CYP3A, CYP1A, and P-glycoprotein levels in healthy volunteers. Gastroenterology 1999;117:89-98.

133. Katiyar SK, Agarwal R, Zaim MT, Mukhtar H. Protection against N-nitrosodiethylamine and benzo[a]pyrene-induced forestomach and lung tumorigenesis in A/J mice by green tea. Carcinogenesis 1993;14:849-55.

134. van Lieshout EM, Bedaf MM, Pieter M, Ekkel C, Nijhoff WA, Peters WH. Effects of dietary anticarcinogens on rat gastrointestinal glutathione S-transferase theta 1-1 levels. Carcinogenesis 1998;19:2055-7.

135. Chou FP, Chu YC, Hsu JD, Chiang HC, Wang CJ. Specific induction of glutathione S-transferase GSTM2 subunit expression by epigallocatechin gallate in rat liver. Biochem.Pharmacol. 2000;60:643-50.

136. Pushparajah DS, Umachandran M, Plant KE, Plant N, Ioannides C. Up-regulation of the glutathione S-transferase system in human liver by

polycyclic aromatic hydrocarbons; comparison with rat liver and lung. Mutagenesis 2008;23:299-308.

137. Teubner W, Fuchs JI, Steinberg P. Enhanced glutathione S-transferase expression in 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine-resistant IEC-18 cells. Cell Biol.Toxicol. 2007;23:153-61.

138. Santana-Rios G, Orner GA, Xu M, Izquierdo-Pulido M, Dashwood RH. Inhibition by white tea of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-induced colonic aberrant crypts in the F344 rat. Nutr.Cancer 2001;41:98-103.

139. Webb LKMFKJKR. Activity of rat UGT1A1 towards benzo[a]pyrene phenols and dihydrodiols. Environmental Toxicology and Pharmacology 2006;21:224-30.

140. Embola CW, Sohn OS, Fiala ES, Weisburger JH. Induction of UDP-glucuronosyltransferase 1 (UDP-GT1) gene complex by green tea in male F344 rats. Food Chem.Toxicol. 2002;40:841-4.

141. Calabrese EJ. Does nutritional status affect benzene induced toxicity and/or leukemia? Med.Hypotheses 1980;6:535-44.

Appendices

Appendix 1: Rat heath assessment sheets for long term study

Health of the animals was monitored by weighing three times a week through, palpation and daily health assessment. Abnormal behavior or symptoms were noted prior to euthanasia. Percent weight loss was calculated at that time for each animal. When calculating % body weight loss in the experimental animals only those with sustained and escalating body weight loss were euthanized as weight loss endpoints.

Date:							
Date arrived	ID	weight	Anorexia	diarrhea	anemia (ears, eyes, nose)	palpable tumour	comments
January 20 2006	C1						
January 20 2006	C2						
January 20 2006	C3						
January 20 2006	C4						
January 20 2006	C5						
January 20 2006	C6						
January 20 2006	C7						
January 20 2006	C8						

January 20 2006	C9						
January 20 2006	C10						
March 21 2006	CGT1						
March 21 2006	CGT2						
March 21 2006	CGT3						
March 21 2006	CGT4						
March 21 2006	CGT5						
March 21 2006	CGT6						
March 21 2006	CGT7						
March 21 2006	CGT8						
March 21 2006	CGT9						
March 21 2006	CGT10						
March 21 2006	CGT11						
March 21 2006	CGT12						
March 21 2006	CGT13						
March 21 2006	CGT14						
March 21 2006	CGT15						
June 6 2006	CGT16						
January 20 2006	HD1						
January 20 2006	HD2						
January 20 2006	HD3						
January 20 2006	HD4						
January 20 2006	HD5						
January 20 2006	HD6						
January 20 2006	HD7						

January 20 2006	HD8						
January 20 2006	HD9						
January 20 2006	HD10						
January 20 2006	HD11						
January 20 2006	HD12						
January 20 2006	HD13						
March 21 2006	HD14						
March 21 2006	HD15						
March 21 2006	HD16						
March 21 2006	HD17						
March 21 2006	HD18						
March 21 2006	HD19						
March 21 2006	HD20						
March 21 2006	HD21						
March 21 2006	HD22						
March 21 2006	HD23						
March 21 2006	HD24						
March 21 2006	HD25						
March 21 2006	HD26						
March 21 2006	HD27						
March 21 2006	HD28						
June 6 2006	HD29						
June 6 2006	HD30						
June 6 2006	HD31						
June 6 2006	HD32						

August 8 2006	HD33						
August 8 2006	HD34						
August 8 2006	HD35						
August 8 2006	HD36						
August 8 2006	HD37						
August 8 2006	HD38						
August 8 2006	HD39						
August 8 2006	HD40						
August 29 2006	HD41						
March 21 2006	HDGT1						
March 21 2006	HDGT2						
March 21 2006	HDGT3						
March 21 2006	HDGT4						
March 21 2006	HDGT5						
March 21 2006	HDGT6						
March 21 2006	HDGT7						
March 21 2006	HDGT8						
March 21 2006	HDGT9						
March 21 2006	HDGT10						
March 21 2006	HDGT11						
March 21 2006	HDGT12						
March 21 2006	HDGT13						
March 21 2006	HDGT14						
March 21 2006	HDGT15						
June 6 2006	HDGT16						

June 6 2006	HDGT17						
June 6 2006	HDGT18						
August 8 2006	HDGT19						
August 8 2006	HDGT20						
August 8 2006	HDGT21						
August 8 2006	HDGT22						
August 8 2006	HDGT23						
August 8 2006	HDGT24						
August 8 2006	HDGT25						
August 8 2006	HDGT26						
August 8 2006	HDGT27						
August 8 2006	HDGT28						
August 8 2006	HDGT29						
August 8 2006	HDGT30						
August 29 2006	HDGT31						
August 29 2006	HDGT32						
August 29 2006	HDGT33						
August 29 2006	HDGT34						
August 29 2006	HDGT35						
August 29 2006	HDGT36						
August 29 2006	HDGT37						
August 29 2006	HDGT38						
August 29 2006	HDGT39						
August 29 2006	HDGT40						
August 29 2006	HDGT 41						

Appendix 2

Appendix 2: Calculation of green tea catechins based on EGCG content

Based on determining the rodent equivalent of the consumption of 1500mL/day

Amount of EGCG in 1500mL green tea
1500mL/day = 20g of dry tea leaves
Green tea contains 13% catechins
1500mL of tea = 2.5 g catechins
The effective dose of green tea contains 2.5g of catechins per day

Amount of EGCG per amount of calories consumed per day
Average adult male consumes 2500Kcal/day
2.5g catechins/2500 Kcal/day
Therefore the average adult male consumes 0.10g of EGCG/100 Kcal of intake.

Amount of calories in 1 kilogram of rat diet
In 1Kg of rat diet there are 160g of fat and 790 g of protein and carbohydrate

160g x 9 Kcal/g = 1440 Kcal coming from fat
790g x 4 Kcal/g = 3160 Kcal coming from protein and carbohydrate
Therefore 1Kg of rat diet contains 4600 Kcal or 460Kcal/100g
100 Kcal = 21.7 g of food

Calculation of Inclusion level of catechins
0.10g of catechins/100 Kcal of intake
100 Kcal = 21.7 g of food

Therefore 0.10g catechins/21.7g of rat food = 0.005 or 0.5% catechins in diet

In order for rats to consume the rodent equivalent of 1500mL of green tea they are required to consume 0.5% catechins per day.

Summary statements for calculation of catechins in diet
HDGT is consumed as 50% of the diet and it must contribute 100% of the catechins to the rat then it needs to contain 1% catechins.

CGT is consumed as 100% of the diet and it must contribute 100% of the catechins to the rat then it needs to contain 0.5% catechins

Appendix 3

Appendix 3: Details of diet formulation

Balancing Amino Acids

Amino acid content of HD and CON diets

PORK HD	g/100g HD	g/500g dry HD	Casein g/200g DM	g/445.139**g Casein (same prot. As HD)	Casein-HD	g/kg Extra-Control Diet	g/kg Extra-HD Diet
Tryptophan	0.28	4.94	2.1	4.67	-0.27	-	-
Threonine	1.00	17.76	6.7	14.91	-2.84	-	-
Isoleucine	1.02	18.22	8.5	18.92	0.70	-	-
Leucine	1.75	31.21	15.4	34.28	3.07	-	-
Lysine	1.96	34.98	13	28.93	-6.04	6.04	-
Methionine	0.58	10.30	4.6	10.24	-0.06	-	-
Cystine	0.28	4.96	3.25	7.23	2.27	-	-
Phenylalanine	0.87	15.52	8.8	19.59	4.06	-	-
Tyrosine	0.76	13.56	9.3	20.70	7.14	-	-
Valine	1.18	21.11	10	22.26	1.15	-	-
Arginine	1.36	24.18	6.4	14.24	-9.93	9.94	-
Histidine	0.87	15.54	4.6	10.24	-5.30	5.30	-
Alanine	1.27	22.66	4.6	10.24	-12.42	-	-
Aspartic acid	2.02	36.08	12.2	27.15	-8.93	-	-
Glutamic acid	3.41	60.90	36.3	80.79	19.89	-	-
Glycine	1.04	18.47	3.2	7.12	-11.35	-	-
Proline	0.88	15.63	20.5	45.63	30.00		21.28

Serine	0.90	16.06	9.7	21.59	5.53	-	-
** DM protein of HD							

Balancing Fatty Acids

Calculation of fatty acids required for HD and HDGT diets										
fatty acids in pork HD	**g/100g HD**	**g/500g dry HD**	**AIN w/ H20**	**g/kg Dry AIN**	**g/500g Diet needed**	**g/kg Fatty acids**	**g/kg Flax**	**g/500g Flax**	**g/kg Safflower**	**g/500g Safflower**
Linolenic (ω3)	0.03	0.54	4.80	5.14	4.60	9.21	16.44	8.22	-9.69	-4.84
Linoleic (ω6)	0.64	11.42	35.70	38.22	26.80	53.60	2.56	1.28	68.41	34.20
Saturated	2.24	39.97	10.80	11.56	-28.41		1.64	0.82	4.24	2.12
Mono-unsaturated	2.93	52.28	16.30	17.45	-34.83		3.00	1.50	9.82	4.91
Total		104.21								
Fat added to Kg of diet: 84.84										

Calculation of fatty acids required for CON and CGT diets									
fatty acids in pork HD	**g/100g HD**	**g/500g dry HD**	**AIN As Fed**	**g/kg DM AIN**	**g/500g Diet needed**	**g/kg Fatty acids**	**g/kg lard**	**g/kg Flax**	**g/kg Safflower**
Linolenic (ω3)	0.03	0.54	4.80	5.14	4.60	5.14	1.20	8.22	
Linoleic (ω6)	0.56	9.99	35.70	38.22	28.23	38.22	12.24		34.20
Saturated	2.24	39.97	10.80	11.56	-28.41	42.91	120.00		

Mono-unsaturated	2.93	52.28	16.30	17.45	-34.83	58.69	120.00		
Fat added to Kg of diet: 162.42									

Balancing Minerals

Calculation of Minerals for HD Diets										
Minerals	**Units**	**g/100g HD AF**	**g/500g dry HD DM**	**AIN g/kg AF**	**g/kg AIN DM**	**mg/500g Min Needed**	**g/kg Min.**	**g/kg Ingredient**	**Mineral Source**	**g/10kg**
Calcium, Ca	Mg	5	89.222	5000	5353.3191	5264.09707	10.528	26.294	Calcium carbonate	262.94
Iron, Fe	mg	0.77	13.74	45	48.179872	34.4396859	0.0689	0.172	Ferric Citrate	1.7203
Magnesium, Mg	mg	22	392.58	513	549.25054	156.673804	0.3133	0.783	Magnesium oxide	7.8259
Phosphorus, P	mg	207	3693.8	3000	3211.9914	-481.798715	-0.9636	0	potassium phosphate monobasic	0
Potassium, K	mg	421	7512.5	3600	3854.3897	-3658.10136	-7.3162	0	potassium citrate, tripotassium monohydrate	0
Sodium, Na	mg	129.173	2305	1039	1112.4197	-1192.59458	-2.3852	0	Sodium chloride	0
Zinc, Zn	mg	1.69	30.157	38	40.685225	10.5281941	0.0211	0.053	Zinc carbonate	0.5259
Copper, Cu	mg	0.058	1.035	6	6.4239829	5.38900785	0.0108	0.027	Cupric Carbonate	0.2692
Manganese, Mn	mg	0.01	0.1784	10	10.706638	10.5281941	0.0211	0.053	Manganous carbonate	0.5259
Selenium, Se	mg	0.0354	0.6317	0.18	0.1927195	-0.43897216	-0.0009	0.000	Sodium selenate anhydrous	0
Chloride	mg	181.991	3247.5	1631	1746.2527	-1501.26695	-3.0025	0.000	NaCl	0

Sulfur	mg	0	0	300	321.19914	321.199143	0.6424	3.4932	Potassium Sulfate	34.932
Iodine	mg	0	0	0.2	0.2141328	0.21413276	0.0004	0.0007	Potassium Iodate	0.0072
Molybdenum	mg	0	0	0.15	0.1605996	0.16059957	0.0003	0.00059	Ammonium paramolybdate 4H20	0.0059
Silicon	mg	0	0	5	5.3533191	5.35331906	0.0107	0.10837	Sodium meta-silicate 9H20	1.0837
Chromium	mg	0	0	1	1.0706638	1.07066381	0.0021	0.021	chromium potassium sulfate 12H20	0.2055
Fluoride	mg	0	0	1	1.0706638	1.07066381	0.0021	0.005	Sodium fluoride	0.0473
Nickel	mg	0	0	0.5	0.5353319	0.53533191	0.0011	0.002	Nickel carbonate	0.0238
Boron	mg	0	0	0.5	0.5353319	0.53533191	0.0011	0.006	Boric Acid	0.0612
Lithium	mg	0	0	0.1	0.1070664	0.10706638	0.0002	0.001	Lithium chloride	0.0131
Vanadium	mg	0	0	0.1	0.1070664	0.10706638	0.0002	0.0005	ammonium vanadate	0.0049
Sub total		0.9687274			16.264165	5.82278729		31.019	Sub total	310.19
									Sucrose	689.81
									TOTAL	1000

Calculation of minerals for CON Diets									
Minerals	**Units**	**g/100g HD**	**g/500g dry HD**	**AIN w/ H20**	**mg/kg Dry AIN**	**g/kg Mineral**	**g/kg Ingredient**	**Mineral source**	**g/10kg**
Calcium, Ca	mg	5	89.222	5000	5353.319	5.35332	13.370	Calcium carbonate	133.6993
Iron, Fe	mg	0.77	13.740	45	48.17987	0.04818	0.292	Ferric Citrate	2.919992
Magnesium, Mg	mg	22	392.577	513	549.2505	0.54925	0.911	Magnesium oxide	9.105612
Phosphorus, P	mg	207	3693.790	3000	3211.991	3.69379	16.229	potassium phosphate monobasic	162.2931
Potassium, K	mg	421	7512.491	3600	3854.39	3.85439	7.888	potassium citrate, tripotassium monohydrate	78.88
Sodium, Na	mg	129.173	2305.014	1039	1112.42	2.30501	5.859	Sodium chloride	58.59213
Zinc, Zn	mg	1.69	30.157	38	40.68522	0.04069	0.078	Zinc carbonate	0.780307
Copper, Cu	mg	0.058	1.035	6	6.423983	0.00642	0.011	Cupric Carbonate	0.11178
Manganese, Mn	mg	0.01	0.178	10	10.70664	0.01071	0.022	Manganous carbonate	0.224035
Selenium, Se	mg	0.0354	0.632	0.18	0.192719	0.00063	0.001512	Sodium selenate anhydrous	0.015116
Chloride	mg	181.991	3247.520	1631	1746.253	1.74625	0.000000	from NaCl	0
Sulfur	mg	0	0	300	321.1991	0.3212	1.7466	Potassium Sulfate	17.46597
Iodine	mg	0	0	0.2	0.214133	0.00021	0.0004	Potassium Iodate	0.003611
Molybdenum	mg	0	0	0.15	0.1606	0.00016	0.00030	Ammonium paramolybdate 4H20	0.002955

Silicon	mg	0	0	5	5.353319	0.00535	0.05418	Sodium meta-silicate 9H20	0.541834
Chromium	mg	0	0	1	1.070664	0.00107	0.010	chromium potassium sulfate 12H20	0.102751
Fluoride	mg	0	0	1	1.070664	0.00107	0.002	Sodium fluoride	0.023666
Nickel	mg	0	0	0.5	0.535332	0.00054	0.001	Nickel carbonate	0.011896
Boron	mg	0	0	0.5	0.535332	0.00054	0.003	Boric Acid	0.03059
Lithium	mg	0	0	0.1	0.107066	0.00011	0.001	Lithium chloride	0.006536
Vanadium	mg	0	0	0.1	0.107066	0.00011	0.0002	ammonium vanadate	0.002458
Sub total						17.939	46.481	Sub total	464.8136
								Sucrose	535.1864
								TOTAL	1000

Balancing Vitamins

Calculation of vitamins for HD Diets							
Vitamins	**Units**	**g/100g HD**	**g/500g HD DM**	**g/100g AIN AF**	**g/kg AIN DM**	**Required vitamins g/500g**	**Required vitamins g/kg Diet**
Thiamin	Mg	0.856	15.27480371	5	5.353319058	-0.009921	-0.01984
Riboflavin	Mg	0.268	4.782298358	6	6.423982869	0.001642	0.003283
Niacin/Nicotinic acid	Mg	5.036	89.86438258	30	32.11991435	-0.057744	-0.11549
Pantothenic acid/Ca pantothenate	Mg	0.766	13.66880799	15	16.05995717	0.002391	0.004782
Folic acid	Mg	0	0	2	2.141327623	0.002141	0.004283
Folate, food	µg	7	124.910778			-0.124911	-0.24982
Folate, DFE	µg_ DFE	7	124.910778	3400	3640.256959	0.003515	0.007031
Vitamin B-12	µg	0.53	9.457530335	25	26.76659529	1.73E-05	3.46E-05
Retinol	µg	2	35.68879372	1200	1284.796574	0.001249	0.002498
Vitamin D	µg	0.2	3.568879372	25	26.76659529	2.32E-05	4.64E-05
Vitamin E (α-tocopherol)	Mg	0	0	50.25	53.80085653	0.053801	0.107602
Vitamin K (phylloquinone)	µg	0	0	900	963.5974304	0.000964	0.001927
Choline	Mg	0	0	1000	1070.663812	1.070664	2.141328
Biotin	Mg	0	0	0.2	0.214132762	0.000214	0.000428
SubTotal						0.944045	1.88809
Sucrose							298.1119
Total							300

Calculation of vitamins for CON Diets						
Vitamins	**Units**	**g/100g HD**	**g/500g HD DM**	**g/kg AIN AF**	**g/kg AIN DM**	**required vitamins for g/kg of Diet**
Thiamin	mg	0.856	15.27480371	5	5.353319058	0.0152748
Riboflavin	mg	0.268	4.782298358	6	6.423982869	0.00642398
Niacin/Nicotinic acid	mg	5.036	89.86438258	30	32.11991435	0.08986438
Pantothenic acid/Ca pantothenate	mg	0.766	13.66880799	15	16.05995717	0.01605996
Vitamin B-6/Pyridoxine	mg	0.47	8.386866524	6	6.423982869	0.00838687
Folic acid	mg	0	0	2	2.141327623	0
Folate, food	µg	7	124.910778			0.12491078
Folate, DFE	µg_ DFE	7	124.910778	3400	3640.256959	0.00364026

Vitamin B-12	μg	0.53	9.457530335	25	26.76659529	2.6767E-05
Retinol	μg	2	35.68879372	1200	1284.796574	0.0012848
Vitamin D	μg	0.2	3.568879372	25	26.76659529	2.6767E-05
Vitamin E (α-tocopherol)	mg	0	0	50.25	53.80085653	0.05380086
Vitamin K (phylloquinone)	μg	0	0	900	963.5974304	0.0009636
Choline	mg	0	0	1000	1070.663812	1.07066381
Biotin	mg	0	0	0.2	0.214132762	0.00021413
SubTotal						1.39154176
Sucrose						298.608458
Total						300

Appendix 4

Appendix 4: Consumption of cooked and cured meat reduces DNA damage in F344 rats

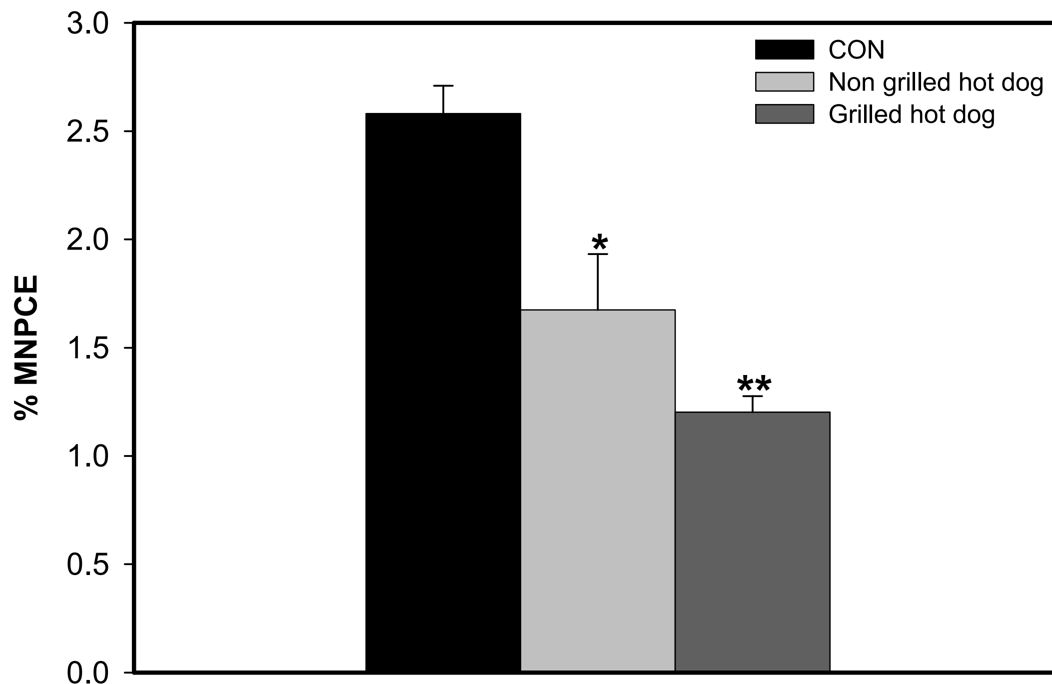

Figure 30: % Micronucleated polychromatic erythrocytes (MNPCE) as a measure of DNA damage. MNPCE are measured using the micronucleus assay. Sixteen days of grilled hot dog or non grilled hot dog feeding followed by 1 day of control diet feeding provided significant protection against ENU (30mg/kg body weight) induced genotoxic damage. One tailed t-test was used to analyze the data. * p>0.05; ** p>0.001. n=8.

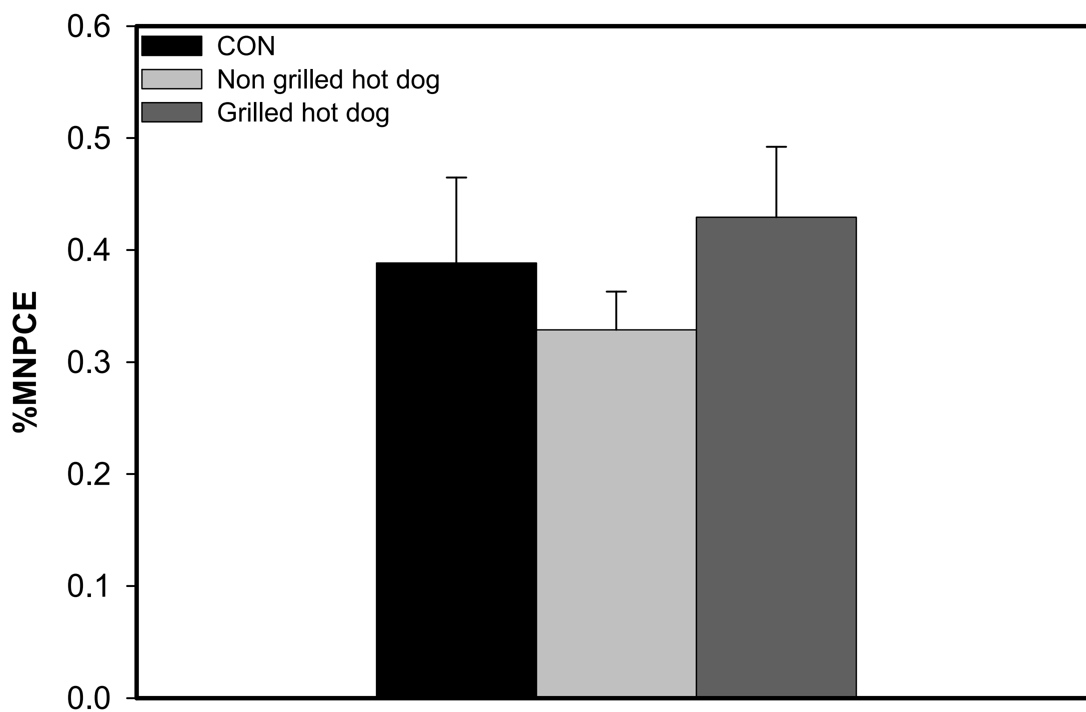

Figure 31: Acute hot dog feeding for 48 hours caused non significant increase in % micronucleated polychromatic erythrocytes (n=5)

Appendix 5