STUDIES ON THE BIOAVAILABILITY, TISSUE DISTRIBUTION, ANTIOXIDANT AND ANTICARCINOGENIC PROPERTIES OF DIETARY LYCOPENE IN RATS

by

Charu K. Jain

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Nutritional Sciences
University of Toronto

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0-612-46129-7
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ABSTRACT
Recent studies have suggested a protective role for lycopene, an antioxidant carotenoid, in the prevention of chronic diseases including cancer. The aim of this thesis was to investigate the bioavailability, tissue distribution, and the in vivo antioxidant and anticarcinogenic properties of lycopene. Rats were maintained on diets containing 10 ppm lycopene. Lycopene was found in all tissues. A significant decrease in serum thiobarbituric acid reactive substances was observed in rats fed the lycopene diet. Incidence of aberrant crypt foci (ACF), induced by azoxymethane in lycopene fed rats showed a trend towards reduced numbers and size but did not reach statistical significance. Based on the results it is concluded that dietary lycopene is absorbed by the rats, distributes to various tissues and acts as an antioxidant in reducing serum lipid peroxides. Trend towards lower incidence of ACF suggest that lycopene may have a protective effect against oxidative stress and colon carcinogenesis.
ACKNOWLEDGMENTS

I want to thank Dr. A.V. Rao for taking me on as his graduate student and allowing me to work in his lab, giving me an opportunity to understand what research is all about. Dr. Rao’s guidance and patience throughout my master’s program is greatly appreciated. Working with Dr. Rao has also been fun. I would like to thank H. J. Heinz Company Canada for generously funding this project. I would also like to thank Dr. D. Yeung, Dr. D.S.R. Sarma and Dr. R. Bruce for their learned advice and their time. I want to thank Sanjiv as he has been an invaluable source of encouragement. His unconditional help and friendship has meant a lot. I also want to thank my lab peers for making my graduate experience enjoyable. Lastly, I want to thank my Mom and dad for their everlasting support and patience. Without their love, little would be possible.
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LIST OF ABBREVIATIONS

AC      Aberrant crypt
ACF     Aberrant crypt foci
AOM     Azoxymethane
GSH     Glutathione
GSX     Glutathione peroxidase
HDL     High density lipoprotein
LDL     Low density lipoprotein
MDA     Malondialdehyde
ROS     Reactive oxygen species
SOD     Superoxide dismutase
TBARS   Thiobarbituric acid reactive substances
VLDL    Very low density lipoproteins
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CHAPTER 1
INTRODUCTION

Cancer is the second major cause of mortality in North America (World Cancer Research Fund, 1997). Among all cancers, colorectal cancer is the second most common cause of death (Pappalardo et al., 1996). Both the incidence and mortality due to colorectal cancer are rising with more than 870,000 new cases diagnosed worldwide since 1996 (World Cancer Research Fund, 1997). As a result, the focus of the scientific community has been towards preventative strategies.

Oxidative stress has been suggested as one of the main mechanisms leading to the development of chronic diseases including cancer (Ames et al., 1995; Halliwell, 1994; Ames et al., 1993a; Ames et al., 1993b). Reactive oxygen species, which are produced endogenously as a result of cellular metabolism as well as arising from exogenous sources, are highly unstable molecules and react readily with other molecules (Trilling and Jaber, 1996; Dreher and Junod, 1996; Stahl and Sies, 1997). Oxidation of important biomolecules such as lipids, proteins and DNA can cause irreversible damage, which may lead to cancer and other degenerative diseases (Ames et al., 1995; Stahl and Sies, 1997; Dreher and Junod, 1996). Although the presence of endogenous antioxidants and enzymes provide a degree of protection against reactive oxygen species mediated oxidative damage of biomolecules, exogenous sources of antioxidants such as the dietary antioxidants are important in minimizing oxidative stress (Stahl and Sies, 1997;
Wiseman, 1996; Rock et al., 1996). Epidemiological studies have indicated that diets high in fruits and vegetables protect against cancer (World Cancer Research Fund; Steinmetz and Potter, 1996). The presence of antioxidants including vitamins, phytochemicals such as the carotenoids, isoflavones, saponins and polyphenols in fruits and vegetables have been suggested as being responsible for these protective effects (Steinmetz and Potter, 1996).

Recent studies have focussed on the role of lycopene, an important carotenoid antioxidant, in the prevention of cancer. The most important sources of lycopene are tomatoes and tomato based products (Rao and Agarwal, 1999; Clinton, 1998). In vitro studies have shown lycopene to be the most efficient singlet oxygen quencher and peroxyl radical scavenger among all carotenoids (Di Mascio et al., 1989; Miller et al., 1996; Mortensen et al., 1997). Support for the in vivo antioxidant properties of lycopene comes from a study performed on healthy human subjects in which biomarkers for serum lipid peroxidation, LDL, protein and DNA oxidation were shown to be reduced after ingesting tomato products for one week (Rao and Agarwal, 1998a; Agarwal and Rao, 1998).

Evidence for an effect of lycopene in reducing cancer development comes from tissue culture, animal and human studies. A recent study found tomato products to be protective against risk of prostate cancer (Giovannucci et al., 1995). Lycopene was suggested as the main component responsible for the protective effect. In another study, lycopene was found to be the only micronutrient in serum associated with a decreased risk for breast
cancer (Dorgan et al., 1998). Very little information is available regarding the role of lycopene in colo-rectal cancer. The colon is a unique organ in that it is exposed to dietary micronutrients from the circulation as well as unabsorbed micronutrients from the lumenal side. It is therefore hypothesized that lycopene may exert a protective effect in the colon from within the colonocyte as well as from the lumenal surface. The overall aim of this thesis is to investigate the absorption, tissue distribution and the effect of dietary lycopene on the incidence of colonic preneoplasia in rats.
CHAPTER 2
LITERATURE REVIEW

2.1 Colon cancer
Cancer is a major cause of death worldwide, and the second leading cause of death in the industrialized countries of the world (Pappalardo et al., 1996). It is predicted that even in developing nations, total mortality due to cancer will double by the year 2015 from 1985 (World Cancer Research Fund, 1997). Among all cancers, colon cancer is the fourth highest prevalent cancer in the world and the second major cause of cancer deaths in North America (World Cancer Research Fund, 1997). Colon cancer occurs at an equal frequency in both men and women (Potter, 1995). In addition to a genetic predisposition to colon cancer, there is also convincing evidence to indicate that the incidence of colon cancer is closely related to lifestyle practices with dietary habits playing an important role (Potter, 1996). The focus therefore has been to look at preventative methods against cancer development.

2.2 Mechanism of cancer development
To understand the role of dietary factors in the causation and prevention of cancer, first the definition of cancer and the overall mechanism of cancer development has to be understood. Cancer is a condition in the body where unregulated cell proliferation leads to the formation of tumors through monoclonal expansion of initiated cells. The most detrimental characteristic of cancer is its ability to invade and metastasize to other sites in
the body where autonomous growth can begin once again (Rubin and Farber, 1988).

As mentioned in the definition, unregulated cellular proliferation is recognized as one the main events in tumorigenesis. In normal cells, cell division and cell death (apoptosis) are in a balance whereas in tumor cells, this balance is disrupted in favor of a phenotype (the result of mutations) with enhanced growth properties (Martin, 1997). This imbalance results in an accumulation of regulatory resistant cells and consequently leading to tumor growth. Additional mutations in tumor cells further enhance their growth potential and may ultimately lead to a malignant phenotype (Macdonald and Ford, 1997).

Although cancer is a multistage process, it is widely accepted as occurring in three main stages: initiation, promotion and progression (Rubin and Farber, 1988; Bird, 1995). Initiation is characterized by alteration of the DNA. A carcinogenic agent, chemical or biological in nature, reacts with DNA altering it such that if the cell is unable to repair the damage, the change becomes irreversible. When the altered cell undergoes cell division, the genetic damage is fixed, or in other words, it has become an initiated cell (Rubin and Farber, 1988). Initiated cells remain quiescent, unless in the presence of a promoter whereby it will be stimulated to undergo cell proliferation. While a promoter is present, the initiated cell multiplies to form microscopic foci (in the case of the colon, aberrant crypt foci), which go on to form larger foci (Bruce et al., 1993). During promotion both cell proliferation and cell death occur, therefore the growth of these preneoplastic lesions is slow (Rubin and Farber, 1988). As the polyps grow into tumors/adenomas,
subpopulations of tumor cells acquire progressively more aggressive growth properties until they are transformed into cells which are capable of autonomous growth (Rubin and Farber. 1988). This is the progression stage of cancer development. These cells no longer require a promoter to stimulate proliferation, acquiring the ability to multiply indefinitely. It takes several years from the initial mutation to a cell till the development of a malignant tumor. When the tumor becomes malignant it is then capable of invading and metastasizing to other areas of the body.

2.3 Oxidative Stress

Oxidative stress has been implicated as playing an important role in many degenerative diseases including cancer, aging and atherosclerosis (Ames et al., 1995; Halliwell, 1994; Ames et al., 1993a; Ames et al., 1993b; Gutteridge, 1995). Oxidative stress is when the balance between reactive oxygen species (ROS) and antioxidants is disrupted such that there is an accumulation of ROS in the body, creating a potential for cellular damage (Trilling and Jaber, 1996; Stohs, 1995).

ROS are reactive chemical species with one or more unpaired electrons in their outer orbit and they have the ability to exist independently (Gutteridge, 1995; Stohs, 1995). These can be of either radical or non radical forms such as superoxide (O$_2^-•$), hydroxyl radical (HO•), hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (¹O$_2$). ROS are generated endogenously from normal cellular metabolic processes such as mitochondrial respiration, (figure 2.1), fatty acid metabolism, cytochrome p450 reactions and the
Figure 2.1. Formation of various free radicals from molecular oxygen (modified from Stohs, 1995).

\[
\begin{align*}
2 \text{O}_2 & \rightarrow 2e^- \\
\rightarrow & 2\cdot\text{O}_2^- \\
\rightarrow & 2\cdot\text{H}^+ \\
\rightarrow & \text{H}_2\text{O}_2 + \text{O}_2 \\
\rightarrow & 2\cdot\text{OH}
\end{align*}
\]
respiratory burst of phagocytic cells as well as from exogenous sources such as ozone, tobacco smoke, ultraviolet light, fatty acids in food (Beckman and Ames, 1998; Dreher and Junod, 1996; Stahl and Sies, 1997; Jacob and Burri, 1996; Stohs, 1995; Benzie, 1996).

The high reactivity of ROS causes them to initiate the oxidative destruction of important biomolecules such as DNA, lipid and protein which if left uncorrected can lead to cellular damage and disturbances in physiological functions (Beckman and Ames, 1998; Benzie, 1996). Oxidative damage to DNA can form mutations and proceed to cancer development (Ames et al., 1993; Trilling and Jaber, 1996). Damage to proteins by ROS can lead to changes in their functional activities thereby influence cellular physiology (Beckman and Ames, 1998; Nakazawa et al., 1996; Burdon, 1995). Oxidative damage to lipids leads to lipid peroxidation and formation of adducts with lipid peroxidation products, contributing to the overall oxidative burden in the cellular environment (Beckman and Ames, 1998; Wiseman, 1996). ROS also influence the cell cycle by modulating the expression and activity of transcription factors, oncogenes and tumor suppressor genes (Wei, 1992; Hainaut and Miller, 1993; Martin, 1997).

A feature of ROS is their ability to cause chain reactions when reacting with lipids and the subsequent production of new radicals as illustrated in figure 2.2 (Rubin and Farber, 1988; Wiseman, 1996; Stahl and Sies, 1997). The reaction of HO• with unsaturated fatty acids causes the abstraction of a hydrogen atom creating a lipid radical, which proceeds to
Figure 2.2. Chain reaction of lipid peroxidation caused by the reactive oxygen species, hydroxyl radical (modified from Rubin and Farber, 1988).
react with molecular oxygen to form a lipid peroxide radical (Rubin and Farber, 1988; Benzie, 1996). Lipid peroxide radicals behave like any ROS, that is, they are themselves capable of initiating chain reactions by removing hydrogen atoms from neighboring phospholipids. The successive formation of more and more lipid peroxide radicals is known as propagation (Benzie, 1996). Lipid peroxidation not only leads to a loss of membrane integrity, but the peroxidation products such as malondialdehyde, can form adducts with important molecules like DNA (Wiseman, 1996; Draper and Hadley, 1990). Lipid peroxidation can also cause considerable damage to membrane proteins which may be biologically more consequential than the oxidative damage of lipids themselves (Gutteridge, 1995).

While ROS are important for signal transduction pathways in the body, they are nonetheless thought to be a pertinent category of carcinogens and are generated in pathological conditions (Suzuki et al., 1996; Jacob and Burri, 1996; Dreher and Junod, 1996; Toyokuni et al., 1995). Biomarkers for oxidized DNA, lipids and protein have been found at high levels in cancer, cardiovascular disease and aging (Ames et al., 1995; Stadtman, 1992; Ames et al., 1993b; Halliwell, 1994). Protein and DNA oxidation was found to be elevated in the aging process (Agarwal and Sohal, 1994; Sohal et al., 1993). Increased malondialdehyde, a lipid peroxidation product, was excreted by premenopausal women with high mammographic tissue densities (Boyd et al., 1995; Boyd and McGuire, 1990). Higher levels of DNA adducts were found in cancer tissues (Musarrat et al., 1996; Wang et al., 1996; Loft and Poulsen, 1996). Compared to controls, subjects with prostate
cancer had increased levels of lipid peroxides (Rigas et al., 1994).

2.4 Antioxidants and oxidative stress

The body defends itself against the damaging effects of ROS through the actions of antioxidants which are both enzymatic and nonenzymatic in nature (Rock et al., 1996; Trilling and Jaber, 1996; Stahl and Sies, 1997). Gutteridge (1995) defines antioxidants as "any substance that, when present at low concentrations, compared with those of the oxidizable substrates, considerably delays or inhibits oxidation of the substrate." These antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase (GSX), bilirubin, glutathione (GSH) and thiols groups, and they protect against cellular damage by scavenging and preventing the formation of ROS (Stahl and Sies, 1997). In addition to endogenous antioxidants, the diet, particularly fruits and vegetables, are an important source of exogenous antioxidants (World Cancer Research Fund, 1997; Rock et al., 1996; Stahl and Sies, 1997; Rousseau et al., 1992). Some examples of dietary antioxidants include ascorbic acid, alpha-tocopherol, carotenoids, flavonoids and polyphenols.

As mentioned earlier, oxidative processes are important for some biological operations, however when the body’s antioxidant defense mechanisms are unable to efficiently control free radicals, the result is tissue damage (Trilling and Jaber, 1996). Therefore, supplementing with antioxidants from exogenous sources may improve the body’s ability to defend against oxidative stress and reduce the incidence of chronic diseases. One of the
dietary classes of antioxidants are the carotenoids.

2.5 Carotenoids

Carotenoids are natural pigments found in plants and microorganisms (Rock et al., 1996). Carotenoids are characterized by their tail to tail bonding of two tetraterpene units where the end groups may be modified to ring structures (Rao and Agarwal, 1999; Stahl and Sies, 1996). Carotenoids also possess an extensive system of conjugated double bonds which account for their antioxidant capabilities (Stahl and Sies, 1996). Approximately 700 naturally occurring carotenoids and their isomers have been identified of which 50 are available for absorption and metabolism through the diet, however, only a selected twenty-one (including seven metabolites) have been found in the bloodstream (Khachik et al., 1995). Protective effects of carotenoids include their exceptional ability to quench singlet oxygen, scavenge free radicals, epithelial cell differentiation through provitamin A activity, increase gap junctional communication through gene expression, inhibit cell proliferation and enhance immune function (Zhang et al., 1991; Zhang et al., 1992). Epidemiological and experimental studies show an association between carotenoid intake and reduced risk for cancer, as well as a better prognosis for individuals who consume a carotenoid rich diet following diagnosis of cancer (Rock et al., 1996). So far, most of the investigation on carotenoids have been focussed on β-carotene. Lycopene is present in abundance in the western diet and possess strong antioxidant ability (Hoffman and Weisburger, 1997). Therefore, studying the bioavailability and anticarcinogenic properties of this carotenoid may prove to be advantageous to human health.
2.6 Lycopene

2.6.1 Properties

Lycopene is a member of the carotenoid family and its various geometric isomers are illustrated in figure 2.3. All-trans lycopene is a linear molecule with a molecular formula of \( \text{C}_{40}\text{H}_{56} \) and a molecular weight of 536.85 daltons. Lycopene is a lipophillic hydrocarbon molecule and insoluble in aqueous solutions (Merck Index, 1983). Lycopene has thirteen double bonds, eleven of which are in a conjugated system (Britton, 1995). Lycopene does not exhibit provitamin A activity since it lacks the \( \beta \)-ionone ring structure which is characteristic in carotenoids that are precursors for vitamin A (Gerster, 1997).

Lycopene is a natural pigment and imparts a red color in the foods in which it is present (Rao and Agarwal, 1999; Nguyen and Schwartz, 1999). In foods, lycopene is found predominantly in its trans form (approximately 95.4% of total lycopene content) whereas serum and tissues contain more cis isomers of lycopene (Krinsky, 1998; Nguyen and Schwartz, 1998; Stahl and Sies, 1992). Stahl and Sies (1992) showed increased cis isomers in tomato juice that was heated. More than 10 cis-lycopene isomers are known to exist, some of which are illustrated in figure 2.3. (Nguyen and Schwartz, 1998). While cis forms of carotenoids tend to be more soluble, and perhaps better absorbed, compared to trans forms (Parker, 1997), the biological significance of different isomers of lycopene are
Figure 2.3. Various isomeric forms of lycopene. Adapted from Nguyen and Schwartz (1999).
not fully understood.

2.6.2 Sources

Lycopene levels in different food sources are listed in Table 2.1 (Rao and Agarwal, 1998). Although lycopene is present in many different fruits and vegetables, it is unique among the carotenoids in that the most predominant source in the human diet comes from tomatoes (Solanum lycopersicum) and tomato-based products such as tomato paste, tomato sauce and tomato juice, accounting for more than 85% of dietary lycopene (Rao and Agarwal, 1999; Clinton, 1998). Lycopene content in different tomato products is shown in Table 2.2 (Rao and Agarwal, 1999). Levels vary from 8.8 μg/g in fresh tomatoes to over 1200 μg/g in tomato powder. The concentration of lycopene in tomatoes varies depending on the variety of tomato, season and ripening stage (Clinton, 1998). The concentration of lycopene in ripe tomatoes ranges from 3.1-7.7 mg/100 grams (Nguyen and Schwartz, 1999).

Although, lycopene is the most abundant carotenoid in tomatoes, other minor carotenoids are also present including 5,6-dihydroxy-5,6-dihydrolycopene, lycopene5,6-epoxide, neurosporene, phytoene, phytofluene, beta-carotene, γ-carotene, δ-carotene, 5,6-dimethoxy-5,6-dihydrolycopene, dimethoxy-prolycopene and lutein (Khachik et al., 1995; Clinton, 1998).

2.6.3 Intake

Lycopene and β-carotene account for 30% each of total carotenoid in the diet (Sies and
Table 2.1. Lycopene concentration in various plant foods (Rao and Agarwal, 1999).

<table>
<thead>
<tr>
<th>Food type</th>
<th>Lycopene level (μg/g wet weight)</th>
</tr>
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<tbody>
<tr>
<td>Tomatoes</td>
<td>8.8 – 42.0</td>
</tr>
<tr>
<td>Watermelon</td>
<td>23.0 – 72.0</td>
</tr>
<tr>
<td>Pink Guava</td>
<td>54.0</td>
</tr>
<tr>
<td>Pink Grapefruit</td>
<td>33.6</td>
</tr>
<tr>
<td>Papaya</td>
<td>20.0 – 53.0</td>
</tr>
<tr>
<td>Apricot</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Table 2.2. Lycopene concentration in various tomato products (Rao and Agarwal, 1999).

<table>
<thead>
<tr>
<th>Tomato product</th>
<th>Lycopene level (μg/g weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh tomatoes</td>
<td>8.8 – 42.0</td>
</tr>
<tr>
<td>Cooked tomatoes</td>
<td>37.0</td>
</tr>
<tr>
<td>Tomato sauce</td>
<td>62.0</td>
</tr>
<tr>
<td>Tomato paste</td>
<td>54.0 – 1500.0</td>
</tr>
<tr>
<td>Tomato soup (condensed)</td>
<td>79.9</td>
</tr>
<tr>
<td>Tomato powder</td>
<td>1126.3 – 1264.9</td>
</tr>
<tr>
<td>Tomato juice</td>
<td>50.0 – 116.0</td>
</tr>
<tr>
<td>Pizza sauce</td>
<td>127.1</td>
</tr>
<tr>
<td>Ketchup</td>
<td>99.0 – 134.4</td>
</tr>
</tbody>
</table>
Stahl. 1998). Consumption of foods containing lycopene is variable depending upon ethnicity and geographic location. Food frequency questionnaire data reveals that, within the United States, individuals of South European origins eat tomato-based foods more frequently than African Americans and Asian Americans, 4.48 servings per week compared to 2.45 and 3.01 servings per week respectively (Giovannucci et al., 1995). The daily intake of lycopene in England is around 1.1 mg/day, whereas in the United States the daily intake is 3.7 mg (Sies and Stahl, 1998). There does not seem to be any toxicity associated with prolonged and a high level of lycopene intake (Reich et al., 1960). Results from our laboratory found approximately 25 mg of lycopene is consumed daily, 50% of which is derived from tomatoes and the remaining amount from various processed tomato sources (Rao et al.. 1999). A food frequency survey found that tomatoes and tomato-based products are the second most consumed vegetable in the United States (Beecher, 1998) thus, the protective effects offered by components in tomatoes, such as lycopene, deserves more attention.

2.6.4 Absorption

Carotenoids, including lycopene, are found in crystalline form or bound protein complexes within foods (Williams et al., 1998; Gerster. 1997; Clinton. 1998). The first step in absorption is releasing lycopene from these bound forms. Processing and the presence of fat in food helps to breakdown cellular matrix and dissolve lycopene, making it available for absorption (Rao and Agarwal, 1999; Johnson, 1998; Williams et al., 1998). Following solubilization in the gut, carotenoids are associated with micelles being
formed in the lumen and get absorbed by intestinal epithelial cells via passive diffusion (Stahl and Sies, 1996; Rao and Agarwal, 1999). Of the total amount of dietary carotenoid ingested, only a small percentage of carotenoid is absorbed by the intestinal epithelium (Williams et al., 1998; Erdman et al., 1993). Once in the intestinal cell, carotenoids get packaged into chylomicrons which are responsible for carrying carotenoids to the bloodstream via the lymphatics (Clinton, 1998). Absorption of lycopene is influenced by several dietary and non-dietary factors some of which include the isomeric form of lycopene, presence of fat and fat analogs, certain dietary fiber, malabsorptive diseases, intestinal parasites (Erdman et al., 1993; Williams et al., 1998).

2.6.5 Factors determining serum/plasma lycopene concentration

Serum concentrations of lycopene are affected by biological and lifestyle factors. Although neither gender, smoking nor alcohol consumption affect plasma lycopene, age is inversely correlated with plasma lycopene (Johnson, 1998; Rao and Agarwal, 1999; Stahl and Sies, 1996). Lycopene levels in women seems to change according to the menstrual cycle, peaking during the middle of the luteal phase (Forman et al., 1996).

The main determinant of serum lycopene is dietary intake of lycopene. After consuming lycopene for one week from a variety of different lycopene rich sources, serum lycopene was found to be increased significantly from all sources in normal healthy human subjects (Rao and Agarwal, 1998a). Plasma lycopene levels rose significantly in a study where healthy males and females consumed a carotenoid rich diet including tomato sauce which
was the main source of lycopene (Yeum et al., 1996). Plasma lycopene increased more than four fold in a volunteer after his diet was supplemented with tomato juice for 19 days (Oshima et al., 1996).

Processing also appears to increase lycopene absorption. Heating tomato juice was shown to increase the uptake of lycopene in human serum compared to unprocessed tomato juice (Stahl and Sies, 1992). Similarly, a comparison between lycopene uptake between tomato paste and raw tomatoes found that lycopene was more bioavailable from the processed tomato form (Gartner et al., 1997).

2.6.6 Lycopene transport in the circulation

Carotenoids are carried in the blood stream by lipoproteins (Nguyen and Schwartz, 1999; van der Berg, 1999). Following a meal, carotenoids are initially found in chylomicrons and very low density lipoproteins (VLDL) (Sies and Stahl, 1998). Their concentration in low density lipoprotein (LDL) and high density lipoprotein (HDL) increase with time and reach peak concentrations 24-48 hours after intake (Sies and Stahl, 1998). Polar carotenoids tend to be carried equally by HDL and LDL whereas lipophillic carotenoids such as lycopene are carried primarily by LDL (approximately 75%) with the remaining 25% by HDL and VLDL (Johnson, 1998). Due to the highly hydrophobic nature of lycopene, it has been hypothesized that it is found mostly in the core of the LDL molecule (Clinton, 1998). Lycopene makes up 21-43% of total plasma carotenoid with a range of 0.22-1.06 nmol/ml (Sies and Stahl, 1998). The half life of lycopene in humans is between
2-3 days (Rao and Agarwal, 1999). There are large differences in the level of lycopene between different tissues within the body indicating that perhaps there is more efficient lycopene transport in some tissues (Nguyen and Schwartz, 1999).

2.6.7 Lycopene as an antioxidant

2.6.7.1 Mechanism

Lycopene is capable of acting as an antioxidant by virtue of its many conjugated double bonds. It is the most efficient neutralizer of singlet oxygen among all carotenoids and has also been found to be a potent scavenger of free radicals (Di Mascio et al., 1988; Rao and Agarwal, 1999; Stahl and Sies, 1996; Gerster, 1997). Neutralization of ROS occurs through both physical and chemical quenching (Stahl and Sies, 1996). Physical quenching, occurring more than 99% of the time, involves energy being absorbed from the excited molecule by the conjugated double bond system and released as vibrational or heat energy (Stahl and Sies, 1996). In this type of quenching, the lycopene molecule retains its form and is available to be used again, whereas in chemical quenching, the reaction is irreversible since lycopene undergoes structural changes (Stahl and Sies, 1996). In the latter reaction, the lycopene molecule reacts with free radicals to form a short lived intermediate species, which later end up as lycopene decomposition products including apocarotenals, apocarotenones and epoxides (Gerster, 1997; Stahl and Sies, 1996).

Being a highly hydrophobic molecule, the greatest scavenging ability of lycopene is seen
in lipophilic environments (Gerster, 1997). After supplementing subjects with lycopene from different dietary sources, serum TBARS (biomarker for lipid peroxidation) were significantly reduced whereas non significant reductions were observed in biomarkers for protein and DNA oxidation (Rao and Agarwal, 1998a). Hence, lycopene may be a biologically important antioxidant by protecting membrane lipids from being oxidized which in turn preserves the integrity of cellular membranes.

2.6.7.2 In vitro studies

Much of the evidence for the antioxidant function of lycopene comes from work done with in vitro systems and virtually all of them indicate lycopene to function as a superior dietary antioxidant. Di Mascio et al. (1989) compared the singlet oxygen quenching ability of various carotenoids, α-tocopherol, bile acids and retinoic acid. They found lycopene to be the most efficient quencher among all, with a greater than two-fold quenching potency compared to β-carotene. A study comparing the radical scavenging ability of nine carotenoids towards 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid diammonium salt (ABTS•⁻ radical cation) using Trolox as the standard, found lycopene to be the most effective scavenger among all carotenoids investigated (Miller et al., 1996). In this study, lycopene was found to be three times as potent at radical scavenging compared to vitamin E. The authors concluded that the conjugated double bond system was responsible for this observation. Similarly, another study which looked at the scavenging ability of various carotenoids against different types of radicals also found lycopene to be an efficient antioxidant (Mortensen et al., 1997). The investigators also
found that lycopene was capable of using different scavenging mechanisms (electron transfer or adduct formation) to neutralize different radical types. Lycopene was the most efficient carotenoid in reducing TBARS formation by 75%, compared to control, in multilamellar liposomes (Stahl et al., 1998). The same study compared the effect of several different mixtures consisting of two carotenoids in reducing TBARS production. They observed that a mixture containing both lycopene and lutein had a synergistic effect in reducing TBARS significantly from the expected calculated value. A study which looked at the relative ability of several antioxidants in reducing carotenoid cations found that lycopene was the most superior carotenoid antioxidant as well as the lycopene cation radical was the most stable carotenoid cation radical (Mortensen and Skibsted, 1997).

2.6.7.3 In vivo studies
Although there is little data on the in vivo effect of lycopene on oxidation, the presence of oxidation products of lycopene in human serum suggests that lycopene is utilized as an antioxidant in vivo (Williams et al., 1998). One of the first in vivo antioxidant human studies was conducted in our laboratory, which looked at the effect of lycopene from several sources on uptake and various in vivo biomarkers of oxidation (Rao and Agarwal, 1998a). Lycopene levels in the serum increased after ingesting tomato juice, spaghetti sauce and oleoresin for one week. This increase in serum lycopene was accompanied by a significant decrease in serum lipid peroxidation as measured by reduced TBARS. Although not significant, there was a trend for decrease in both serum protein and DNA oxidation. It was hypothesized that one reason for the lack of significance could be due to
the short treatment time of only one week. LDL oxidation was also found to be reduced using two different methods to detect lipid peroxidation (Agarwal and Rao, 1998). In a study using just one human subject, LDL was enriched with carotenoids from tomato juice for 19 days and after photoirradiation of serum samples, carotenoid enriched LDL showed significantly less cholesteryl ester hydroperoxides compared to non supplemented LDL (Oshima et al., 1996). Another study conducted in our laboratory looked at the effect of withdrawing lycopene containing foods from the diet on serum lycopene levels and lipid peroxidation and also the effect of oxidation from smoking on serum lycopene and lipid peroxidation (Rao and Agarwal, 1998b). Two weeks on a lycopene free diet reduced serum lycopene by 50% and concurrently serum TBARS were raised by 25%. After three cigarettes, smokers had a 40% decrease in serum lycopene and at the same time a 40% increase in serum TBARS.

2.6.8 Anticarcinogenic properties of lycopene

Research in the area of anticarcinogenic properties of lycopene is fairly new. However, the evidence in support of lycopene is accumulating. This evidence comes from different lines of investigations including tissues culture, animal and epidemiological studies and was recently reviewed (Rao and Agarwal, 1999; Giovannucci, 1999).

2.6.8.1 Epidemiological studies

Most epidemiological work as related to cancer and carotenoids in the past have looked at the association of β-carotene on cancer risk. Other carotenoids have been largely ignored
until recently. A recent review on the effect of tomato or lycopene consumption or blood lycopene on cancer risk of several different cancers indicated, for the most part, that lycopene possesses anticancer properties (Giovannucci, 1999). A prospective cohort study looking at intake of various fruits and vegetables against the risk of prostate cancer in a relatively healthy population of men, found that only lycopene among all carotenoids (α-carotene, β-carotene, β-cryptoxanthin and lutein) had a significant inverse trend associated with risk for prostate cancer (Giovannucci et al., 1995). Neither α-carotene, β-carotene, cryptoxanthin nor lutein showed any association. Also, among 46 fruits and vegetables, only four (out of which three were tomato products) were related to a decreased risk for prostate cancer. This significant trend was more pronounced for advanced prostate cancer cases consuming ten or more servings of tomato or tomato products per week compared to less than one and a half servings per week. Another study which looked at several case control studies cases of digestive tract cancers and the intake of tomatoes, found protection in individuals with highest intake of tomatoes compared to the lowest intake for upper digestive tract cancers, stomach, colon and rectal cancers (Franceschi et al., 1994). Another prospective cohort study looking at various serum micronutrients and the risk of breast cancer found that only lycopene was inversely associated with risks (Dorgan et al., 1998). With increasing serum lycopene, there was a decrease in breast cancer risk while other micronutrients (β-cryptoxanthin, lutein/zeaxanthin, α-tocopherol, β-carotene, retinol, selenium and α-carotene) showed either marginal or no association for breast cancer risk. In a case control study looking at the dietary intake and serum concentrations of various carotenoids and risk of cervical
intra-epithelial neoplasia, investigators found that after adjusting for confounding variables, only lycopene (both dietary and serum) were inversely associated with cervical intra-epithelial neoplasia (VanEenwyk et al., 1991). Another cervical dysplasia study done only with black women carried some suggestion that higher intake of lycopene and higher serum lycopene may protect against dysplasia (Kanetsky et al., 1998).

2.6.8.2 Tissue culture studies

Lycopene suppressed cell growth of Ishikawa cells (a human endometrial cancer cell line) after 24 hours compared to α-carotene and β-carotene (Levy et al., 1995). The same investigators also found that lycopene reduced cell proliferation of human endometrial, mammary and lung cancer cell lines at lower concentrations compared to α-carotene and β-carotene while growth of normal fibroblasts remained unaffected. A decrease in gap junctional communication is thought to be a condition promoting tumor formation, thus enhancing or upregulating gap junctional communication is thought to inhibit tumor formation (Krutovskikh et al., 1997). Lycopene treatment was found to upregulate gap junctional communication in C3H/10T1/2 mouse embryo fibroblast cell line by increasing the expression of the gene connexin43 which encodes for important gap junctional proteins (Zhang et al., 1991). In a different study using the same cell line, lycopene was shown to inhibit chemically induced malignant transformation of cells (Bertram et al., 1991). Treatment with lycopene of a human leukemia cell line not only cause a 40% decrease in cell growth, but also potentiated the differentiation of leukemia cells when added along with retinoic acid (Countryman et al., 1991; Bankson et al., 1991). Lycopene
reduced cell injury and death in rat hepatocytes after administration of carbon tetrachloride, a liver toxin (Kim, 1995). Lycopene also protected mouse hepatocytes against microcystin-LR, a liver tumor promoter (Matsushima-Nishiwaki et al., 1995).

2.6.8.3. Animal studies

Animal experiments provide good models to perform studies under controlled conditions and are frequently used to carry out cancer bioassays. At a dose of 5 mg/kg body weight of lycopene, α-carotene and β-carotene for 5 days, gap junctional communication in rat liver were found to be enhanced (Krutovskikh et al., 1997). In an earlier study, 500 μg of lycopene was found to improve resistance of mice against a virulent strain of bacterial infection, better than β-carotene (Lingen et al., 1959). The effect of lycopene was dose dependant. The authors found that the protective effect of lycopene occurred only when it was in the animal system for 14 hours or longer. Lingen et al. (1959) also found that lycopene treatment resulted in an increase in the percentage of mice surviving after inoculation with Ehrlich ascites tumor. Feeding rats a 300 ppm lycopene diet significantly reduced the size and liver volume fraction occupied by chemically induced (by DEN) liver preneoplastic foci (Astorg et al., 1997). No effect was seen with vitamin A or the other carotenoids in this study. In a spontaneous mammary tumor model, 0.5 ppm of lycopene significantly delayed the onset as well as inhibited mammary tumor development (Nagasawa et al., 1995). After 10 days of treatment with 0.5 ppm lycopene diet, immune response improved accompanied by an increase in helper T cells and a normalization of intrathymic T cell differentiation in a spontaneous model of mammary
tumor development (Kobayashi et al., 1996). In a chemically induced mammary tumor model (using DMBA as the carcinogen), intraperitoneal injections with lycopene at 10 mg/kg body weight twice a week for 18 weeks, significantly decreased the number of tumors and also reduced the area of tumors compared to controls while β-carotene showed no protective effect (Sharoni et al., 1997). Rats given lycopene in the form of tomato juice at 25 ppm for twelve weeks showed a significant decrease in the multiplicity of urinary transitional cell carcinomas (Okajima et al., 1998). Lycopene dissolved in drinking water at 50 ppm was given to rats for 21 weeks and was shown to significantly reduced the incidence and multiplicity of both lung adenomas and carcinoma compared to control (Kim et al., 1997). Although the role of lycopene in several cancers using animals have been reported, few studies have looked at the effect of lycopene on colon cancer and results from these studies show inconsistent findings (Narisawa et al., 1996: Kim et al., 1997).

2.6.8.4 Human studies

Most researchers looking at the effect of carotenoids in human cancer have concentrated on supplementing with β-carotene, either alone or with other dietary antioxidants, and the results have been inconsistent. Individuals supplemented with β-carotene, vitamin E and selenium in China had a decrease in mortality from cancer (Blot et al., 1993). In the Polyp Prevention Study Group, there was no difference in new adenoma reoccurrence in individuals supplemented with β-carotene, vitamin E and vitamin C (Greenberg et al., 1994). Supplementing with β-carotene had significantly increased the incidence of lung
cancer in male smokers from those not receiving β-carotene (The Alpha-Tocopherol. Beta Carotene Cancer Prevention Study Group, 1994). Exposure to sunlight and near UV light may play a role in skin cancer development through the action of ROS (Chatterjee et al., 1990). No intervention studies with lycopene have been conducted as of yet.

Serum and tissue lycopene levels were found to be significantly lower in prostate cancer patients compared to age matched controls (Rao et al., 1999a). No significant differences were observed between cases and controls for any other carotenoid investigated. One study which looked at a single, large dose of β-carotene on serum lycopene and β-carotene levels as well as the effect on skin levels of these two carotenoids after irradiating with UV light, found a significant decrease in skin lycopene concentration while there was no change in skin β-carotene, suggesting a preferential use of lycopene over β-carotene as an antioxidant (Ribaya-Mercado et al., 1994).
CHAPTER 3
HYPOTHESIS AND OBJECTIVE

3.1 Rationale
The role of lycopene in colon cancer has not been studied well. The few laboratory investigations into the effect of lycopene on colon cancer have used greater than dietary doses and have turned out inconsistent results. Colon cancer is a prevalent disease worldwide with rising incidence and mortality rates (World Cancer Research Fund, 1997). It occurs at an equal frequency in both men and women (Potter, 1995). In the United States, colon cancer is the second major cause of death due to cancer with a correlative survival rate of less than 50% (Pappalardo et al., 1996). Among all cancers, it is one that is highly correlated with diet (World Cancer Research Fund, 1997). Therefore, focussing on preventative strategies with emphasis on dietary intervention may be beneficial. Besides endogenous production of reactive oxygen species, the colon is an organ exposed to oxidative stress from dietary factors and the activity of intestinal microflora (Stone and Papas, 1997). It is also a site for unabsorbed food constituents. Hence, the colon is unique in that it may be exposed to protective dietary elements from both the circulation as well as unabsorbed constituents from the lumen.

3.2 Hypothesis
Dietary lycopene, after being absorbed, functions as an in vivo antioxidant and protects against lipid, protein and DNA oxidative damage and thereby reduces the risk of cancer.
However, the possibility of other mechanisms can not be ruled out. This is illustrated in figure 3.1.

3.3 **Objective**

3.3.1 **Overall**

To study the protective role of dietary lycopene in azoxymethane induced colonic preneoplasia in rats.

3.3.2 **Specific objectives**

a) To assess the stability of lycopene in rodent diet

b) To evaluate the effect of feeding lycopene feeding on food intake and growth in rats.

c) To assess the bioavailability of dietary lycopene

d) To measure tissue distribution of lycopene in rats

e) To evaluate the in vivo antioxidant properties of lycopene

f) To study the effect of dietary lycopene on the incidence of colonic preneoplasia

g) To investigate the role of lycopene in initiation and promotional stages of colonic preneoplasia in rats.
Figure 3.1. Hypothetical role of the effect of dietary lycopene on cancer risk.
CHAPTER 4

STABILITY OF LYCOPENE IN THE DIET

4.1 Introduction

Lycopene is the most efficient singlet oxygen quencher in the carotenoid family (DiMascio et al., 1989). Due to the photosensitivity of lycopene, diets were prepared in multiple, small batches to avoid its loss. Also, care was taken so that preparation and storage of the diet did not result in lower lycopene level from the initial concentration. These measures included preparing the lycopene diet under diffused light during all steps of preparation, storing the diet in an opaque container in 4 °C cold storage, minimizing exposure of the diet to light in foodcups by inserting an opaque sleeve, and replacing diet in foodcups frequently. The dietary concentration of lycopene administered to rats in all the studies was 10 ppm. This level was chosen since it is equivalent to two servings of tomato products per day, a level that is achievable through dietary means. To ensure the level of lycopene in the diet, batch variability and its stability, diets were analyzed for their lycopene content.

4.2 Materials and methods

4.2.1 Diets

AIN93M powder diet and soya oil, stripped of antioxidants, were received packed separately (Dyets Inc., Bethelhem, Pennsylvania). Composition of the diet is shown in appendix A. Diets were made in 5 kg batches, sufficient for 1 1/2 weeks. To avoid lycopene
degradation, diets were stored in opaque plastic containers and kept in cold storage at 4°C.

4.2.2 Lycopene

Tomato oleoresin containing 6% lycopene (LycoRed Natural Products Industries Ltd., Beer Sheva, Israel) was used as the source of lycopene. Composition of the oleoresin is shown in appendix B.

4.2.3 Lycopene oil preparation

To arrive at a final lycopene concentration of 10 ppm, 835 mg of the tomato oleoresin containing 6% lycopene was weighed out in a glass beaker covered with aluminum foil under subdued light. The oleoresin was first dissolved in 60-80 ml of petroleum ether and then solubilized in 200 g of soya oil by evaporating the petroleum ether from the oil mixture under nitrogen gas and in a warm water bath for 30-40 minutes. Care was taken to minimize exposure to light during all operations.

4.2.4 Diet preparation

Soya oil (containing lycopene or free of lycopene) was slowly added to 4800 grams of powdered AIN93M diet. Contents were mixed at low speed (position 1) for 5 minutes using a Hobart’s Mixer (Hobart Canada, North York, Ontario). After loosening by hand, the diet was set to mix for an additional 10 minutes at a higher speed (position 2). Aliquots of 2-3 grams of the diet were taken to perform lycopene analysis and the
remaining diet was stored in opaque plastic containers and kept in cold storage at 4°C to protect against photo-oxidation. Preparation of the lycopene diet was done under diffused light.

4.2.5 Lycopene stability

In all foodcups containing the lycopene supplemented diet, an opaque, plastic sleeve approximately 4mm in thickness was inserted to protect the diet from light. One batch was chosen to perform lycopene stability analysis in both the foodcup and the coldroom. Starting from the date of preparation (day 0) and days 1, 2, 3, 4, 5, 6, 11, 14 and 28, 2 gram samples were taken each time from both foodcup and cold storage to perform lycopene analysis.

4.2.6 Lycopene analysis

Approximately 300 mg of diet was weighed out in 50 ml Teflon tubes. 2 ml deionized water was added to dissolve the powdered diet. 20 ml of extraction mixture (BHT in petroleum ether: acetone: ethanol in a 2:1:1 ratio) was added to tubes, vortexed and set on an automatic shaker for 15 minutes. 5 ml distilled water was added to tubes and shook for an additional 5 minutes on the shaker. Tubes were then allowed to stand for 5 minutes at room temperature for phase separation. The top layer was collected and the optical density was measured at 472 nm using extraction mixture as the blank. Absorbance for each sample was done using six repeats. Concentration of lycopene was assessed in ppm using an extinction coefficient value for 1% solution as 3450 cm⁻¹.
4.3 Results

Lycopene supplemented diet at a concentration of 10 ppm gave a peach-like color. A total of twelve batches of 5 kg lycopene supplemented diets were made for the entire study period for all experiments. The concentration of lycopene in each batch is listed in table 4.1. Lycopene levels ranged from 7.82 ± 0.18 ppm to 10.39 ± 0.27 ppm. The average lycopene concentration in the diet for the twelve batches was 8.8 ± 0.33 ppm. Absorbance at 472 nm for the control diet was negligible, as expected; indicating there was no lycopene in the control diet.

The stability of lycopene in the foodcup and coldroom over time is illustrated in Figure 4.1. Lycopene concentration remained stable for a period of 28 days in both foodcup and the cold storage. Starting at an initial lycopene concentration of 8.63 ± 0.22 ppm, the level of lycopene did not fall below 7.77 ± 0.05 ppm and 7.75 ± 0.30 ppm during the 28 day period in the foodcup and coldroom respectively.

4.4 Discussion

Lycopene is a carotenoid and a natural pigment and it is responsible for the red color seen in tomatoes. The peach color of the lycopene supplemented diet is likely due to the lycopene from the oleoresin and no other carotenoid since more than 96% of the total carotenoid contribution in the tomato oleoresin was lycopene. On average, lycopene concentration from the several diet batches was calculated to be 8.8 ppm and ranged from
Table 4.1. Lycopene concentration in different batches of diet. Results are expressed as mean ± SD.

<table>
<thead>
<tr>
<th>Batch #</th>
<th>Lycopene concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.46 ± 0.44</td>
</tr>
<tr>
<td>2</td>
<td>8.78 ± 0.57</td>
</tr>
<tr>
<td>3</td>
<td>10.39 ± 0.60</td>
</tr>
<tr>
<td>4</td>
<td>8.39 ± 0.15</td>
</tr>
<tr>
<td>5</td>
<td>9.42 ± 0.40</td>
</tr>
<tr>
<td>6</td>
<td>9.98 ± 0.49</td>
</tr>
<tr>
<td>7</td>
<td>8.20 ± 0.17</td>
</tr>
<tr>
<td>8</td>
<td>8.63 ± 0.49</td>
</tr>
<tr>
<td>9</td>
<td>7.82 ± 0.40</td>
</tr>
<tr>
<td>10</td>
<td>8.94 ± 0.37</td>
</tr>
<tr>
<td>11</td>
<td>8.78 ± 0.37</td>
</tr>
<tr>
<td>12</td>
<td>8.57 ± 0.24</td>
</tr>
<tr>
<td>control</td>
<td>Not detected</td>
</tr>
<tr>
<td>Average</td>
<td>8.86 ± 0.74</td>
</tr>
</tbody>
</table>
Figure 4.1. Lycopene concentration over 28 days in foodcup at 25°C and coldroom at 4°C. Solid line, foodcup; broken line, coldroom.
7.82 to 10.39 ppm. Thus, in the remainder of this thesis, when discussing the level of lycopene administered, it will refer to 10 ppm. During preparation of the diet, heating the oleoresin in soya oil for 40 minutes did not result in a decrease in the concentration of lycopene from the targeted 10 ppm level, suggesting that lycopene is resistant to mild heating. The reproducibility of diet preparation is seen by the similar lycopene levels from batch to batch. These results also indicate that the lycopene in the original tomato oleoresin remained stable for the entire experimental period.

Lycopene was found to be stable for 28 days at room temperature in animal foodcups and at 4°C in the coldroom. Indicating that the use of plastic sleeves in foodcups was effective in protecting against degradation of lycopene in diet from light. With the finding that lycopene was stable for 28 days, the frequency of changing the diet in foodcups (every 3-4 days) and preparation of new diets (every 1½ - 2 weeks) was judged to be satisfactory.

A 10 ppm concentration of lycopene in the diet was chosen because it represents a level which is achievable through the diet alone. 10 ppm is equivalent to 2 servings of tomato or tomato products per day and is in accordance with current dietary recommendations for healthy eating (Health Canada, 1992).
CHAPTER 5
THE EFFECT OF DIETARY LYCOPENE ON FOOD INTAKE AND GROWTH OF RATS

5.1 Introduction

In order to assess the effect of lycopene on biomarkers for oxidation and colon cancer in rats, it was necessary to establish that feeding lycopene did not cause adverse effects on rats or confound results. Hence, food intake and growth of rats were measured to ascertain the tolerance of rats to 10 ppm lycopene diet.

The proportion of lycopene absorbed from the diet has not been reported previously. In humans, it is estimated that between two and fifty percent of dietary β-carotene is absorbed. the variability resulting from a variety of dietary and non-dietary factors (Erdman et al., 1993). Therefore, the second objective was to estimate the net lycopene uptake from a 10 ppm diet in rats. To assess net lycopene absorption, fecal output along with food intake data had to be collected.

Apart from feeding a 10 ppm lycopene diet to rats, another variable in this thesis was the use of a colon specific carcinogen, azoxymethane (AOM) to induce colon cancer. Therefore, calculations for the effect of lycopene on food consumption, body weight and fecal output for both carcinogen and non-carcinogen treated animals was performed.
5.2 Materials and Methods

5.2.1 Body weight

Weekly body weights of all rats were taken using an electronic balance. Values reported are taken when rats began the experimental period, after azoxymethane injections, until the time they were sacrificed. To assess whether lycopene had an adverse growth effect on rats, comparisons were made between four experimental groups:

(i) Control group
(ii) Lycopene treated animals
(iii) Control plus carcinogen treatment
(iv) Lycopene plus carcinogen treatment

The statistical test consisted of comparing the slopes of all four groups together after performing a linear regression test in the Graph Pad program, version 2.0 (GraphPad Software Inc.). A p-value less than 0.05 was considered statistically significant.

5.2.2 Food intake

Food consumption was measured for the four groups of rats (same as in body weight measurements) for one week during three time periods during the course of the experiment; at the beginning, middle and end. A mean weekly value for each rat for all three time periods was calculated. A two way ANOVA test was performed in Microsoft Excel 97 program. Values are reported as mean ± SEM. A p-value less than 0.05 was considered statistically significant.
5.2.3 Output and lycopene levels in rat fecal pellets

Fecal pellets were collected for two days from three rats in each of the four above mentioned groups at an age of twenty-one weeks. Weight of the pellets was taken with an electronic balance.

To analyze the lycopene level in fecal pellets, approximately 300 mg of mortar ground feces was weighed out in 50 ml Teflon tubes. The remaining steps of the method is the same as in Chapter 4 (section 4.2.6). Absorbance for each sample was done using four repeats.

5.2.4 Lycopene consumption

Total lycopene intake was assessed by calculating the amount of lycopene (in micrograms) in the amount of food consumed by rats using the dietary lycopene concentration (in ppm). The amount of lycopene (in micrograms) in feces was deducted from this value to arrive at the net lycopene uptake. Students t-test (Microsoft Excel 97) was used. All values are reported as mean ± SEM.

5.3 Results

Daily food intake during the beginning of the study was 16.0 ± 0.3, 14.5 ± 0.3, 15.6 ± 0.3 and 15.5 ± 0.3 grams for control, lycopene fed, control and AOM injected, and lycopene fed and AOM injected groups respectively (figure 5.1). There were no statistical differences in food intake between the four groups (p > 0.05). Similarly, there were no
Figure 5.1. Food intake of animals during the beginning, middle and end of experimental period. Control, control group; Lycopene, lycopene fed group; Con+AOM, control and AOM injected animals; LP+AOM, lycopene fed and AOM injected group. Values are expressed as mean ± SEM, n=12.
significant differences in food consumption between groups during the middle and end of the experimental period. The distribution of mean values of diet consumed by rats ranged from 14.5 – 17.4 grams per day however, on average, rats consumed 16.3 grams of food daily.

During the fourteen week experimental period, rats in all four groups (control, lycopene, control and AOM, and lycopene and AOM) had continuous weight gain as illustrated in figure 5.2. After performing a linear regression, the $r^2$ was found to be greater than 0.95 for all four curves, indicating a good fit with data from the fourteen week period. Rats from all groups grew at a similar rate since a comparison of the slopes for the four curves yielded a p-value greater than 0.05. The average weight gain of rats in this study was 9.9 grams per week.

Mean daily fecal output was $2.19 \pm 0.15$, $1.72 \pm 0.07$, $2.06 \pm 0.15$ and $2.38 \pm 0.15$ grams for control, lycopene fed, control and AOM injected, and lycopene fed and AOM injected groups respectively. The differences in fecal output between groups was not significantly different ($p > 0.05$). Average daily fecal output was 2.09 grams. Total lycopene consumed by non carcinogen and AOM treated rats were $138.08 \pm 6.10$ and $146.71 \pm 4.31$ µg per day, of which $36.75 \pm 4.97$ and $40.10 \pm 7.15$ µg were excreted, respectively. Therefore, net lycopene uptake by rats was $101.33 \pm 11.07$ µg for non carcinogen treated rats and $106.61 \pm 6.06$ µg for AOM treated animals. There were no statistical differences ($p > 0.05$) between groups for all lycopene measurements. Average daily lycopene
Figure 5.2. Weight gain of animals over 14 weeks. Open squares, control group; filled triangles, lycopene fed group; open diamond, control and AOM injected animals; filled circles, lycopene fed and AOM injected group. Values are expressed as mean ± SEM, n=12.
consumption, excretion and absorption for rats was 143.47, 38.84 and 104.63 μg respectively (figure 5.3).

5.4 Discussion

Lycopene at a concentration of 10 ppm was well tolerated by rats as seen by similar food consumption and the steady growth of animals. There were also no interactive effects seen between lycopene and AOM in growth and food intake of rats. On average, rats ate 16 grams of food per day. The range of food eaten by young adult rats is between 10 – 20 grams daily (Canadian Council on Animal Care, 1993). Therefore, the rats in this study were eating a normal, healthy amount of diet.

The absorption of carotenoids like β-carotene are thought to range from 2 - 50 % with the variability as a result of several dietary and non dietary factors (Erdman et al., 1993; Williams et al., 1998). Approximately 143 μg of lycopene was ingested daily by rats, from which 105 μg was absorbed. This corresponds to a net uptake of 73 % lycopene from the diet, which is higher than the reported range and represents extremely efficient absorption. It is thought that carotenoid absorption is enhanced by sufficient digestion of food matrix or processing and also by adequate dietary triglycerides in order to have micelle formation in the intestinal lumen (Parker, 1997). When the level of lycopene from several commercial tomato products were analyzed, it was found that lycopene levels was higher in all tomato products compared to raw tomatoes (Tonucci et al., 1995). Lycopene fed to healthy humans in the form of either fresh tomatoes or tomato paste, caused a 2.5
Figure 5.3. Average daily consumption, excretion (in feces) and net uptake of lycopene in rats from 10 ppm lycopene diet. Values are expressed as mean ± SEM, n=8.
fold higher increase in serum lycopene from tomato paste compared to tomatoes (Gartner et al., 1997).

The high uptake of lycopene in rats in this study may be because the experimental conditions provided for optimal absorption. Firstly, the food given to rats was in a powdered form, which means the particle size was very fine and considerably improved absorption of lycopene. Secondly, the source of lycopene was a tomato oleoresin, which already underwent processing allowing lycopene to break free from protein complexes in food matrix, implying that more lycopene was available for absorption. Also, the major component of the oleoresin were triglycerides, indicating that lycopene was to some degree already solubilized in oil. In addition to this, the oleoresin was heat processed with soya oil for 30 – 40 minutes (chapter four) which allowed for lycopene to further solubilize in the oil phase of the diet and be more readily available for absorption. Heat processing is thought to improve the bioavailability of carotenoids, by rupturing the walls of plants and releasing the carotenoid (Erdman et al., 1993). Serum lycopene rose only in subjects who consumed tomato juice which had been heated for one hour compared to subjects drinking non heated tomato juice (Stahl and Sies, 1992).

The presence of interfering factors, such as certain dietary fibers, may inhibit the amount of carotenoid absorbed by intestinal mucosa (Parker, 1997). The absence of any such impeding factors may have also facilitated the net absorption of lycopene in rats. To summarize, the combined effect of the factors mentioned above contributed to the high
uptake of lycopene from the diet. In conclusion, lycopene from oleoresin and fed to rats at a concentration of 10 ppm is not toxic and does not cause adverse effects in physiological parameters in male rats.
6.1 Introduction

It is important to evaluate the concentration of dietary lycopene in tissues in order to assess whether sufficiently high levels are reached during treatment and then in turn to assess whether these levels are adequate for lycopene to function as an in vivo antioxidant. Data on lycopene distribution in tissues is limited. Most of this data pertains to human tissues and very little on lycopene accumulation in animal tissues. It is essential to determine lycopene distribution in animal models since they are often used when performing cancer bioassays. Most animal studies, which have looked at lycopene concentration in tissues, have used very high doses (Zhao et al., 1998; Lingen et al., 1959).

Protective effects against cancer from tomato and tomato products consumption can be seen at dietary levels in epidemiological studies (Giovannucci et al., 1995; Franceschi et al., 1994). Thus the objective of this study was to measure the absorption and tissue distribution of lycopene administered at a dietary dose (10 ppm) in several organs in rats.

It has been hypothesized that oxidative stress and reactive oxygen species play an important role in cancer and other chronic diseases (Ames et al., 1995; Halliwell, 1994). Products and outcome of lipid and protein oxidation are implicated in several pathologic conditions including cancer (Wang et al., 1996; Boyd et al., 1990; Kadota et al., 1991).
Breakdown of lipid peroxides results in the formation of highly reactive products such as malondialdehyde, which can bind to proteins leading to pleiotropic effects including disruption of membrane-bound protein/receptor functioning which may be important in various disease states (Wiseman, 1995; Draper and Hadley, 1990). They can also react with nucleic acids leading to mutagenicity (Mukai et al., 1975). The antioxidant function of lycopene has been explored in vitro systems and it has been found that lycopene is the most efficient quencher of singlet oxygen and a potent radical scavenger (Di Maschio et al., 1989; Miller et al., 1996; Mortensen et al., 1997). However, there is little data on the in vivo antioxidant effect of lycopene and virtually no data from animal studies to substantiate in vitro findings. Thus, the second objective of the study was to examine the effect of dietary lycopene on in vivo oxidation by measuring biomarkers of lipid and protein oxidation in rats.

6.2 Materials and methods

6.2.1 Animals

Male Fischer rats (F344) at six weeks age weighing approximately 185 grams from Harlan Sprague Dawley, Inc. Indianapolis, Indiana were used in the study. Animals were housed singly in plastic cages with corncob bedding under controlled temperature (22°C) and humidity (50%) conditions. Animals were maintained under a 12-hour light and dark cycle spanning from 7 p.m. to 7 am. All rats ate and drank ad libitum. At the end of the experiment, rats were killed via cervical dislocation. Animals were cared for according to the guidelines under the Canadian Council on Animal Care. The protocol was approved
by the Ethics Committee for the use of animals, University of Toronto.

6.2.2 Study design

The same animals were used for assessing tissue distribution of lycopene and its effect on in vivo oxidation. Twelve rats were randomly divided into two groups containing equal number of animals receiving either lycopene supplemented diet (10 ppm) or control diet. All rats were initially acclimitized for a total of two weeks starting with laboratory Purina chow for one week followed by AIN93M diet for the second week. After the acclimitization period, rats began eating their respective diets. Food intake and weekly body weights were measured. The study period lasted 8 weeks. At the end of the experimental period, animals were killed and blood and tissues were collected.

6.2.3 Blood and tissue collection

Blood samples were collected in vacutainers at the end of the experiment by cardiac puncture and processed to obtain serum. Animals were dissected and the following tissues were collected; prostates, colons, livers, spleens, hearts, lungs and brains. All tissues were washed with 0.9% saline, blot dried weighed and minced. The samples were then stored in vials at -70°C until further biochemical analyses.

6.2.4 Lycopene estimation

The level of lycopene in serum and tissues was estimated using reverse phase HPLC method.
6.2.4.1 Lycopene estimation in serum

400 µl of 2-propanol was added to 400 µl of serum in glass tubes and vortexed. Two milliliters of extraction mixture (hexane: methylene chloride, 5:1, v/v) with 0.015% butylated hydroxytoluene (BHT) was then added to the tubes, vortexed and allowed to incubate for one hour in the dark at room temperature. During the one hour incubation period, tubes were vortexed twice. Tubes were then centrifuged at 4°C for 10 minutes at approximately 2000 rpm. 1 ml of the upper layer was collected and dried with nitrogen gas. dissolved in 40 µl of methylene chloride containing BHT and resuspended in 160 µl of mobile phase (methanol: acetonitrile: methylene chloride: water, 700:700:200:16, v/v). Lycopene was analyzed using reverse phase HPLC using VYDAC 201HS54 column (The Separations Group. Hesperia, CA) and an absorbance detector at 472 nm with a flow rate of 1 ml/min. An external lycopene standard (Sigma Chemical Co., St. Louis, MO) was used to identify and quantify lycopene peaks (Rao and Agarwal, 1998a; Stahl et al., 1992).

6.2.4.2 Lycopene estimation in tissues

Tissue aliquots were dissolved in 200 µl saturated NaOH solution and incubated overnight at 37°C. 100 µl methanol, 100 µl acetone and 2 ml of extraction mixture (0.015% BHT in hexane: methylene chloride, 5:1, v/v) were added to tubes and vortexed. The remainder of the procedure was identical to serum lycopene analysis described above.
6.2.5 Oxidation assays

In vivo lipid peroxidation was measured in serum using the thiobarbituric acid reactive substances (TBARS) spectrophotometric assay and in vivo protein oxidation was measured using the DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)) assay to measure reduced thiols respectively.

6.2.5.1 TBARS in serum

Serum was allowed to thaw completely at room temperature. 10 μl of BHT solution was added to 100 μl of serum in glass tubes and vortexed. 700 μl of 1.0% orthophosphoric acid was added followed by 200 μl of 0.6% 2-thiobarbituric acid (TBA) solution, and then vortexed. Tubes were then incubated in a hot water bath at 90 °C for 45 minutes. After the incubation period, tubes were cooled in a cold water. 1.0 ml of n-butanol was added to tubes, vortexed and centrifuged at 2000 rpm for 10 minutes. The upper layer was aspirated and its absorbance measured on a spectrophotometer at 535 nm against a blank (Rao and Agarwal, 1998a; Jentzsch et al., 1996). The assay was performed as five repeats for each sample. TBARS were calculated in μM using the extinction coefficient, 1.56 X 10^5 M^1 . cm^1 (Jentzsch et al., 1996).

6.2.5.2 Total reduced thiols in serum

Serum was allowed to thaw completely at room temperature. 150 μl of 0.25 M Trizma Base buffer at pH 8.2 was added to 50 μl of serum and vortexed in plastic vials. 10 μl of
10 mM DTNB solution and 790 µl of methanol were added and vortexed after each addition. Vials were then allowed to incubate for 15 minutes at room temperature. Using a Beckman micro-centrifuge, vials were centrifuged for 5 minutes at 10,000 rpm. The optical density of the supernatant was read using a spectrophotometer against a blank at 412 nm (Rao and Agarwal, 1998a; Hu, 1994). The assay was performed as five repeats for each sample. Reduced thiols were calculated as µM using extinction coefficient value of 13,600 M⁻¹·cm⁻¹ (Hu, 1994).

6.2.5 Statistical analysis

All statistical analyses were done using two-tailed students t-test in the Excel 5.0 program (Microsoft Corp., USA). P values of less than 0.05 were considered statistically significant. Results are expressed as mean ± SEM.

6.3 Results

Throughout the study, all animals remained healthy and behaved normally. Body weights and food intake were similar for lycopene supplemented and control groups (see chapter 5).

Lycopene concentration in serum and tissue samples is shown in table 6.1. Serum and all tissues from lycopene fed rats showed the presence of lycopene whereas no lycopene was
Table 6.1 Concentration of lycopene in serum and tissues of rats after being on a lycopene supplemented diet at a concentration of 10 ppm for 8 weeks and 5 days. Values are expressed as mean ± SEM per milliliter serum and mean ± SEM per gram wet weight for tissues for 6 rats.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Lycopene concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>19.55 ± 3.02 ng/ml</td>
</tr>
<tr>
<td>Spleen</td>
<td>11.39 ± 1.19 µg/g</td>
</tr>
<tr>
<td>Liver</td>
<td>10.90 ± 1.02 µg/g</td>
</tr>
<tr>
<td>Prostate</td>
<td>0.173 ± 0.027 µg/g</td>
</tr>
<tr>
<td>Lung</td>
<td>61.98 ± 8.10 ng/g</td>
</tr>
<tr>
<td>Heart</td>
<td>42.75 ± 15.46 ng/g</td>
</tr>
<tr>
<td>Colon</td>
<td>24.90 ± 3.05 ng/g</td>
</tr>
<tr>
<td>Brain</td>
<td>9.24 ± 3.19 ng/g</td>
</tr>
</tbody>
</table>
detected in serum or tissues from animals in control group within the minimum detection level of the assay. The concentration of lycopene in tissues ranged from microgram to nanogram amounts with the lowest level in brain (9.24 ± 3.19 ng/g wet weight) and the highest in the spleen and liver (11.39 ± 1.19 and 10.9 ± 1.02 μg/g wet weight respectively). Prostate tissue also contained considerably higher levels of lycopene than the colon, lung and heart. These values represent non-fasting samples.

Figure 6.1 illustrates the effect of 10 ppm dietary lycopene on serum TBARS and thiols. There was a significant reduction (p<0.02) in serum lipid peroxidation as measured by the 14% decrease in TBARS in lycopene fed animals (9.14 ± 0.23 μmol TBARS/L) compared to controls (10.68 ± 0.40 μmol TBARS/L). Dietary lycopene also increased thiols in serum of lycopene fed rats (491.96 ± 24.98 μmol/L) by 8% over the control group (453.09 ± 63.51 μmol/L), indicating a decrease in protein oxidation.

6.4 Discussion

Dietary lycopene at a 10 ppm concentration had no adverse effects on rats as seen by comparable food intake and growth rate of animals between control and lycopene supplemented groups. Lycopene was absorbed and accumulated in tissues. Compared to the majority of animal studies, where lycopene has been administered at very high doses, in this study, lycopene was detected in serum and tissues at a much lower dose. The high concentration of lycopene observed in the prostate is in agreement with previous studies suggesting that lycopene tends to accumulate in tissues such as prostate, adrenals and
Figure 6.1. The effect of dietary lycopene on serum lycopene, TBARS and thiols. L-, control group; L+, lycopene fed animals. The asterisk indicates a statistically significant lower value compared to control at p < 0.02. Values are expressed as mean ± SEM, n=6.
testes (Clinton et al., 1996). So far, there has been no reports on lycopene from any part of the central nervous system, however, we detected very low levels of lycopene in the brain.

The few animal studies that have measured lycopene in tissues have found that liver lycopene levels tend to be higher than levels in other tissues (Lingen et al., 1959; Sharoni et al., 1997; Zhao et al., 1998). Likewise, this study found liver to be one of two tissues with the highest lycopene content (spleen being the other tissue). The liver is the first organ in the body to receive nutrients after their absorption from the gut, which may account for the very high concentration of lycopene seen in this tissue.

Lycopene levels in other animal tissues are scarce to lacking in the literature and values that are available, do not compare to levels from this study. For instance, mice given a single, large, intraperitoneal dose (1 mg) of lycopene, accumulated 14 μg and 7 μg of lycopene in spleen and lung tissue respectively (Lingen et al., 1959). Concentrations for both tissues in this study are considerably lower than the mice study. In a dose response study, rats consumed lycopene supplemented diets with the lowest lycopene dose at 50 ppm and the highest at 1240 ppm (Zhao et al., 1998). Lycopene content ranged from 47-97 ng/g for prostate, 134-375 ng/g for lung and 152-308 ng/ml for serum. At 10 ppm, prostate lycopene level was higher than prostate levels resulting from all dietary doses of lycopene from Zhao et al. (1998) study, however, lung and serum levels were much lower. It is difficult to draw comparisons from other animal studies due to differences in species (i.e. mice or rat) amount of lycopene administered, method of administration and
duration of treatment. More tissue distribution studies must be performed in animals before arriving at standard tissue values.

The varying concentrations of lycopene in different tissues, 1000 fold difference between the lowest value (in the brain) and the highest value (liver and spleen), suggests selective uptake of the carotenoid and that perhaps a tissue specific mechanism is involved. Carotenoids are carried within the blood via lipoproteins, particularly in LDL (Erdman et al., 1993). It is thus conceivable that tissues high in LDL receptors selectively accumulate lycopene.

The antioxidant properties of lycopene are maintained in vivo as seen by the statistically significant reduction in lipid peroxidation and a trend for reduced protein oxidation. These results are in agreement with a human study recently published which showed that dietary lycopene from various sources fed for one week, protected against lipid, protein and DNA oxidation in serum (Rao and Agarwal, 1998). It has been hypothesized that lipid and protein oxidation play an important role in cancer and other chronic diseases.

Polyunsaturated fatty acids are susceptible to lipid peroxidation and their degradation leads to production of new ROS during chain reactions, interaction of lipid degradation products with cellular macromolecules, loss in membrane fluidity, altered membrane potential, loss of channel function, increased permeability to ions such as H+ and Ca2+, which may ultimately lead to cell destruction (Gutteridge, 1995; Nakazawa et al., 1996; Stohs, 1995). Oxidative damage to proteins can cause amino acid modification,
aggregation, fragmentation, activate latent enzymes and inactivate enzymes (Nakazawa et al., 1996; Stohs, 1995).

An important decomposition product of lipid peroxides is malondialdehyde (MDA) (Benzie, 1996). Lipid peroxidation was estimated as TBARS, which are indicative of MDA, a mutagen and carcinogen (Mukai and Goldstein, 1975). MDA is highly reactive and capable of reacting with nucleic acids, proteins and phospholipids (Draper and Hadley, 1990). Putative MDA adducts were found to accumulate more in breast tissue of cancer patients than in non-cancer controls (Wang et al., 1996). Applying MDA onto mice skin in the presence of a promoter produced a high incidence of skin tumors (Draper and Hadley, 1990). A criticism of the TBARS method is that it also measures MDA formed during the acid heating phase of the assay, however this was minimized by adding 0.015% BHT to samples to prevent auto-oxidation of lipids. Also, for improved quantification of MDA, n-butanol was used to extract the MDA-TBA product.

When proteins are exposed to oxidative stress, their sulfhydryl groups are easily oxidized, so protein oxidation can be measured as the loss of sulfhydryl or thiol groups (Hu, 1994). Serum thiol groups are often diminished in people suffering from a variety of chronic diseases (Agarwal and Sohal, 1994; Baker and Wood, 1991). Total sulfhydryl groups in serum of patients with coronary artery disease was lower than in controls, and this was more prominent with the severity of coronary artery diseases (Kadota et al., 1991). Results from this study show that lycopene at dietary doses is bioavailable and gets
distributed to various tissues in the body and acts as an in vivo antioxidant as seen by the reduction in lipid peroxidation and protein oxidation.
CHAPTER 7
THE EFFECT OF DIETARY LYCOPENE ON COLONIC PRENEOPLASIA

7.1 Introduction
Colon cancer is the second cause of death due to cancer in North America and its incidence and mortality rates are rising (Pappalardo et al., 1996). Once colon cancer has been diagnosed, the five-year relative survival is approximately 50% (World Cancer Research Fund, 1997; Pappalardo et al., 1996). Among all cancers, colon cancer is one that is highly influenced by the diet and has a great potential to be prevented (Potter, 1996). In fact it is thought that diet may account for up to 90% of geographical differences in colo-rectal cancer incidence (Pappalardo et al., 1996; World Cancer Research Fund, 1997). Consumption of tomatoes and tomato products, which contain lycopene, a carotenoid antioxidant, as well as circulatory levels of lycopene are shown to be associated with a decreased risk of various types of cancers in a number of epidemiological studies (Franceschi et al., 1994; VanEwyck et al., 1991; Giovannucci et al., 1995; Dorgan et al., 1998). Animal studies have reported on the anticarcinogenic properties of lycopene. After ascites tumors were transplanted in mice, animals treated with lycopene had better survival rates than control mice (Lingen et al., 1959). Both, spontaneous and chemically induced mammary tumor development was significant reduced in animals receiving lycopene treatment (Nagasawa et al., 1995; Sharoni et al., 1997). Lycopene treatment also resulted in a decreased risk for liver, bladder and lung cancer compared to control animals (Astorg et al., 1997; Okajima et al., 1998; Kim et al.,
In chapter six of this thesis, lycopene at a dietary dose of 10 ppm was found to distribute in colonic tissue and it also decreased in vivo serum lipid peroxidation. Translating the antioxidant aptitude of lycopene to its anticarcinogenic potential, has not been well studied. Experimental work on the effect of lycopene in colon carcinogenesis is sparse and results are inconsistent. Hence the aim of this study was to investigate the effect of dietary lycopene on the incidence and size of colonic preneoplastic lesions. Colon cancer is a good model to study the effect of dietary lycopene because it is unique in that it is exposed to both circulatory and lumenal dietary lycopene. In the model used to study colon carcinogenesis, aberrant crypt foci (ACF) were used as endpoints using the colon specific carcinogen AOM. ACF are commonly accepted precursor lesions for colonic tumors (Bruce et al., 1993; Bird, 1995).

7.2 Methods and materials

7.2.1 Animals

See Chapter 6 (section 6.2.1).

7.2.2 Study design

Forty-eight male F344 rats were acclimatized for a total of two weeks, beginning with laboratory Purina rat chow for the first week and then on powdered AIN93M diet (Dyets Inc., Bethelhem, PA) for the second week. The animals were then randomized into four groups containing 12 rats each. The four groups were as follows (figure 7.1):

(i) A group fed control diet (AIN93M),
Figure 7.1. After acclimatization (ACC), rats were divided into four groups (n=12) receiving either control or lycopene supplemented (10 ppm) diets and given either saline or azoxymethane (AOM, 15 mg/kg body weight) i.p. injections. Colons were removed from animals at the end of 14 weeks from time of injections and scored for ACF.
(ii) A group fed a 10 ppm lycopene supplemented diet (AIN93M + lycopene from tomato oleoresin, see chapter 3),

(iii) A group fed control diet and receiving azoxymethane (AOM) injections, and lastly

(iv) A group fed lycopene supplemented diet and receiving AOM injections.

After the acclimitization period, rats ate their respective diets for 2 weeks then received either AOM or saline injections, and continued on their diets. Food intake, fecal weights and weekly body weights were measured. The study was terminated 100 days after AOM injections by killing rats via cardiac puncture and cervical dislocation under halothane.

Blood and colons were collected.

7.2.3 AOM preparation

AOM was used as the colon specific carcinogen (Sigma Chemical Co., St. Louis, MI). It was dissolved in 0.9% saline to a concentration of 5 mg/ml a day prior to injections. Single intraperitoneal injections of AOM were given at a dose of 15mg/kg.

7.2.4 Colon preparation

Colons were removed from rats, washed free of fecal material, rinsed with 0.9% saline, cut longitudinally and spread out on filter paper. They were fixed in 10% buffered Formalin (Fisher Scientific, Fair Lawn, NJ) between two filter papers for a minimum of one week before enumeration.
7.2.5 ACF enumeration

The method as described by Bruce et al. (1993) and Mclellan E., (1990), was used to enumerate ACF. Fixed colons were stained with 0.2% methylene blue in Kreb’s Ringer bicarbonate buffer in a petri dish for 15-25 minutes and mucosal surfaces were examined with a light microscope under 40X magnification. The number of ACF and AC were recorded. Enumeration began from the rectal end up to the cecum. ACF were identified from normal crypts by their darker stain, enlarged and slightly elongated size, enlarged and slightly elongated cryptal opening, thicker epithelium and larger pericryptal zone. Total number of ACF and AC were recorded for all colons. Total ACF were divided into three groups to reflect foci of different aggressive states, foci consisting of 1-3 AC’s (small), 4-6 AC’s (medium) and 7 or more AC’s (large).

7.2.6 Statistical analysis

All statistical analyses were done using unpaired two-tailed students t-test in the Excel 5.0 program (Microsoft Corp., Redmond, WA). P values of 0.05 were considered statistically significant. Results are expressed as mean ± SEM.

7.3 Results

Body weights, food intake and fecal output in all four groups were similar (refer to chapter 5). The specific lycopene uptake for the two groups supplemented with lycopene diet was 101.33 ± 11.07 µg and 106.61 ± 6.06 µg lycopene per day for AOM free animals and AOM injected animals respectively. This value was calculated using food intake and
fecal output data. The comparable levels of net lycopene intake in these two groups suggest that AOM had no affect on the absorption of lycopene from the diet nor did it affect the amount of lycopene excreted in feces.

The effect of 10 ppm dietary lycopene on AOM induced ACF incidence is shown in figure 7.2. Saline injected animals (animals from both control and lycopene supplemented diet groups) showed negligible ACF development, consequently they are not depicted in figure 7.2, whereas AOM injected animals developed an average of 260 ± 27 ACF. Although statistically significant differences in ACF or AC between lycopene fed and control animals were not observed, some interesting trends were noticed. Dietary lycopene caused a 6.7% reduction in AOM induced ACF incidence over the control group (242 ± 29 and 260 ± 27 respectively). Lycopene treated and saline injected animals also did not show any ACF development. Total AC development in lycopene fed animals (590 ± 67) was also suppressed compared to controls (613 ± 47).

When ACF was classified according to their size (1-3 AC’s or small, 4-6 AC’s or medium and 7 ≥ AC’s or large), every class of foci size from lycopene treated animals showed a decrease in the occurrence of ACF compared to control animals. This difference is better depicted as percent reduction from control (figure 7.3). There was a progressively greater reduction in ACF incidence in lycopene supplemented rats going from small to medium to large sized foci, corresponding to a 5.8%, 12.9% and 20.3%
Figure 7.2. Effect of 10 ppm dietary lycopene on AOM induced ACF and AC in rats. L-, control diet fed; L+, lycopene fed. Values are mean ± SEM, n=12.
Figure 7.3. Percent ACF decrease in lycopene fed animals from controls. ACF were classified according to their size as small (AC/ACF<4), medium (AC/ACF=4-6) and large (AC/ACF>6). n=12.
reduction respectively. It should be noticed however, medium and large sized foci respectively represent 16% and 3% of the ACF population.

7.4 Discussion

Lycopene fed to rats at 10 ppm for 100 days was well tolerated as seen by similar food intake, fecal output and weight gain (chapter five). The presence of AOM did not affect the uptake of lycopene in animals since groups receiving AOM injection or not had comparable lycopene uptake levels.

Results from this study indicate that there was a trend towards reduced AOM induced incidence of ACF in lycopene fed animals. During the process of colon carcinogenesis, ACF appear in the early stages and sequentially develop into polyps and adenomas and eventually into carcinomas, and thus ACF can be used reliably as end point biomarkers of colon cancer for short term animal studies (Bruce et al., 1993). In this study, although there is indication of a decrease in ACF incidence, the results were not statistically significant. However, lycopene fed animals consistently showed a lower incidence of AC and ACF. Suppression of the growth of cancer cell lines in tissue culture and growth of tumors in animal models through lycopene supplementation has been illustrated in various studies. In comparison to the concentrations used in other animal models, 10 ppm represents a low level of administration. Astorg et al 1997, found that lycopene at 300 ppm significantly reduced the size of chemically induced liver preneoplastic foci. A significant reduction in the incidence and multiplicity in lung adenomas and carcinomas
was observed in male mice at 50 ppm but not 25 ppm (Kim et al, 1997). Similarly, Okajima et al 1998, had observed a significant decrease in the number of transitional cell carcinomas in rats given tomato juice at a concentration of 25 ppm. At 10 ppm dietary lycopene concentration, liver was found to have one of the highest levels of lycopene (see chapter six). Lung was also shown to have a significant amount of lycopene. However, compared to these two organs, colon shows very little lycopene accumulation. The above mentioned studies found significant protection against liver and lung cancer development with high lycopene feeding. Perhaps, increasing the concentration of lycopene in rodent diet would result in a higher level of lycopene in the colon, reaching closer to levels found in lung and liver, and this may consequently provide better protection against ACF development.

In this study, both incidence and size of colonic preneoplastic lesions were decreased with lycopene feeding. Protective effects of lycopene were more noticeable in larger foci (ACF consisting of a greater number of aberrant crypts). Larger foci are thought to have more growth advantage over smaller foci, hence they are more aggressive (Bird, 1995). Aggressive foci may be regulated and respond differently to growth factors or promoters than less aggressive foci allowing them to get promoted more rapidly to the next step in carcinogenesis. While the decrease in total ACF in lycopene supplemented animals is modest, the pronounced reductive effect seen in ACF of increasing size suggests that lycopene may slow the growth of ACF detaining them from growing into bigger foci. From these results it appears that lycopene at a dietary dose, reduced the incidence of
ACF indicating the potential of lycopene in lowering the risk of colon cancer.
CHAPTER 8
THE EFFECT OF DIETARY LYCOPENE ON DIFFERENT STAGES OF
COLONIC CARCINOGENESIS

8.1 Introduction

Lycopene was found to be an effective antioxidant in in vivo studies (Rao and Agarwal, 1998a). Oxidative stress has been implicated as playing a role in cancer development (Halliwell, 1994; Ames et al., 1995). When dietary lycopene was fed to rats (chapter 7), there was indication that it reduced the incidence and size of colonic preneoplastic lesions. Colon cancer is a multistep process, which is commonly described as occurring in three stages, initiation, promotion and progression (Bruce et al., 1993; Bird, 1995). Oxidative stress may be present in more than just the initiating event of tumourogenesis. It has been hypothesized that there is a chronic presence of oxidative stress in cancer (Toyokuni et al., 1995). Therefore, lycopene acting as an antioxidant could hypothetically provide beneficial or protective effects during all stages of cancer (figure 8.1). The aim of this study was to determine which phase (i.e., initiation or promotion) of the cancer process lycopene was exerting its protective effect. During initiation a normal cell after reacting with a carcinogen, undergoes irreversible damage acquiring selective growth advantage over normal cells. Promotion is the stage where initiated cells undergo cellular proliferation (Pitot, 1993; Rubin and Farber, 1988; Weinstein, 1998). To observe the effect of lycopene during initiation, it was administered for a short time, i.e. during the initiation event (i.e. carcinogen injection). Whereas to see the effects during promotion.
Figure 8.1. Proposed mechanism for the role of lycopene in inhibiting oxidative damage in multistep carcinogenesis.
lycopene was given after initiation.

8.2 Materials and methods

8.2.1 Animals

See Chapter 6 (section 6.2.1).

8.2.2 Study design

Thirty-six male F344 rats were randomly divided into three groups containing 12 rats each (figure 8.2). All rats were acclimatized for two weeks starting with laboratory Purina chow for the first week and AIN93M diet for the last week. One group received control diet (control), one group received lycopene diet (10 ppm) one week prior to and after AOM injections (defined as the Initiation group) and the last group received control diet one week prior to and after AOM injections (defined as the Promotion group). One week after AOM injections (given intraperitoneally as a single dose of 15 mg/kg body weight), rats in the initiation and promotion groups switched diets for the remainder of the study. Food intake, fecal weights and weekly body weights were measured. The study was terminated 100 days after AOM injections by killing rats via cardiac puncture and cervical dislocation under halothane. Blood and colons were collected.

8.2.3 AOM and colon preparation

AOM was used as the colon specific carcinogen (Sigma Chemical Co., St. Louis, MI). It was dissolved in 0.9% saline to a concentration of 5 mg/ml a day prior to injections.
Figure 8.2. Schematic of experimental design. Animals were randomized into three groups (C, I, P; n=12) and all received azoxymethane (AOM, 15 mg/kg body weight) injections. Groups were: C, animals fed control diet throughout experimental period; I, animals received lycopene supplemented diet (10 ppm) one week before and after AOM injections then fed control diet; P, animals fed control diet one week before and after AOM injections then fed lycopene supplemented diet. Colons were removed and scored for ACF after 14 weeks from AOM injections. C, control group; I, initiation group; P, promotion group.
Single intraperitoneal injections of AOM were given at a dose of 15mg/kg. Colons were removed from rats, washed free of fecal material, rinsed with 0.9% saline, cut longitudinally and spread out on filter paper. They were fixed in 10% buffered Formalin (Fisher Scientific, Fair Lawn, NJ) between two filter papers for a minimum of one week before enumeration.

8.2.4 ACF enumeration

See Chapter 7 (section 7.2.5). Total ACF were divided into two groups to reflect foci of different aggressive states AC/ACF<4 (small) and AC/ACF>4 (large).

8.2.5 Statistical analysis

All statistical analyses were done using one way ANOVA in the sigmastat program, version 2.0. P values of less than 0.05 were considered statistically significant. Results are expressed as mean ± SEM.

8.3 Results

Growth, food consumption and excretion were similar in animals among all three groups (refer to chapter 5).

The effect of 10 ppm lycopene on ACF incidence and size is illustrated in figure 8.3. Lycopene given during initiation or promotion resulted in decreased total ACF and AC. Dietary lycopene when given during the promotion stage inhibited ACF incidence by
Figure 8.3. Effect of dietary lycopene during the initiation and promotion stages of AOM induced ACF and AC development. C, control; I, initiation; P, promotion. Values are mean±SEM, n=12.
17% whereas ACF inhibition was 11% when lycopene was fed during the initiation stage. Similarly, the suppressive effect of dietary lycopene on total AC was larger during the promotion stage (17% inhibition) than during the initiation stage (5% inhibition). These results were not statistically significant.

Results for when ACF were divided into smaller (1-3 AC’s) and larger (4+ AC’s) sized foci are illustrated in figure 8.4. Both the initiation group and promotion group show a decrease in foci consisting of 1-3 AC’s from control animals, with the promotion group showing a slightly larger decrease compared to the initiation group, 15.5% and 12.9% respectively. This pattern is repeated when looking at foci containing 4 or more AC’s, where again, the promotion group shows a greater reduction in ACF from controls than the initiation group, corresponding to a 22.5% (promotion) decrease against 2.2% (initiation). However, these results did not reach statistical significance.

8.4 Discussion

Feeding 10 ppm dietary lycopene showed a trend towards reduced ACF incidence and size in both initiation and promotion groups suggesting that lycopene may play a protective role in both phases of cancer. However, the protection was greater during the promotional phase than during the initiation phase (a reduction in ACF incidence of 17% compared to 11% respectively). While the results did not reach statistical significance, definite trends were observed. The promotion group consistently showed a decline in both incidence and size of ACF. A hallmark of promotion is cellular proliferation. After
Figure 8.4. Comparison of percent decrease in initiation and promotion from control. ACF were classified as small (AC/ACF<4) or large (AC/ACF≥4). n=12.
initiation, initiated cells undergo clonal expansion (proliferation) to form foci (preneoplastic lesions) (figure 8.4). Further and continuous proliferative stimulus causes foci to grow into larger foci and tumors (adenomas) of increasing size and eventually to carcinomas. This entire growth process of tumors is categorized into the promotional stage of cancer. Thus, the greater protection offered to lycopene supplemented rats in the promotional group is possibly due to decreased cell proliferation. In a tissue culture study, the authors found that lycopene suppressed proliferation of cancer cell lines more efficiently than other carotenoids investigated (Levy et al., 1995).

Genetic and phenotypic differences among ACF imply that they are a biologically heterogeneous population. While some ACF may undergo regression or remodelling, others persist and develop more rapidly. Aggressive ACF possess the advanced growth features required to persevere and continue along the stepwise process towards tumorigenesis. ACF of increasing size are thought to be more aggressive as instanced by the increase number of ACF with greater than three AC’s after administering a known tumor promoter to rats (Bird, 1995).

When we divided foci to reflect different sizes, we observed the greatest reduction of ACF (22 %) in larger foci in the promotion group. Therefore, it would seem that lycopene perhaps slows the growth of aggressive foci. Conversely, in the initiation group where rats exposed to lycopene for two weeks only at the start of experimentation, the protection offered by the lycopene diet declines from the smaller foci to larger foci. Therefore, it
appears that the presence of lycopene creates an environment that is not as favorable for the growth of aggressive foci compared to when it is not present. The ramification of these results is that lycopene may retard the growth of tumors and perhaps even the onset of cancer. This may explain the observation made by Nagasawa et al. (1995), where they found the incidence of spontaneous mammary tumors in lycopene supplemented mice to be delayed by two months. Giovannucci et al. (1995) also reported that increased consumption of tomatoes and tomato products was more protective against advanced staged prostate cancer.

Since the initiation group shows a reduction in ACF incidence compared to control animals, and the promotion group shows a decrease in ACF incidence from both control and initiation groups, thus it would be expected that if lycopene was given during both initiation and promotion phases, an even greater reduction would be observed. However, this was not seen. In fact, when lycopene was given throughout both periods (refer to chapter 5), the inhibition was less than both initiation and promotion groups. The reason for this observation is unclear and unexplainable.

Lycopene is a superior antioxidant and in the human diet is found almost exclusively from tomato and tomato products. Since oxidative stress is implicated in the cancer process, measures to reduce oxidative damage could prove to be beneficial. Since there is constant oxidative stress during progression of cancer (Toyokuni et al., 1995), lycopene acting as an antioxidant would be expected to provide maximum protection against tumor
growth during promotional stages. Therefore, tomato and tomato products should perhaps be included in the diet for healthy eating and disease prevention.
CHAPTER 9
GENERAL DISCUSSION

9.1 Discussion

The overall objective of this study was to investigate the role of lycopene, a carotenoid antioxidant present in tomatoes and tomato products, on colon cancer in rats. To accomplish this objective, the following specific objectives were undertaken: to determine the stability of lycopene under the conditions of this study, the absorption and tissue distribution of lycopene, its effect in in vivo oxidation, and its effect on chemically induced colon carcinogenesis in rats. Male Fischer (F344) rats were used. A colon specific carcinogen, azoxymethane (AOM) was used to induce preneoplastic lesions of colon cancer in rats. Serum and tissue lycopene levels were measured to assess its absorption and tissue concentrations. Lipid and protein oxidation was measured in serum to assess the in vivo antioxidant property of lycopene. Aberrant crypt foci (ACF) were measured as makers of colonic preneoplasia.

Lycopene from oleoresin was found to be stable in cold storage and foodcups of animals for the parameters of this study. Dietary lycopene administered at a concentration of 10 ppm to Fischer (F344) male rats was found to be well tolerated. Feeding of lycopene caused lycopene to distribute to serum and several tissues which was accompanied by a decrease in in vivo biomarkers of oxidation in serum. Dietary lycopene also showed a trend toward reduced incidence and size of colonic preneoplastic lesions, particularly
larger or more biologically aggressive lesions.

The source of lycopene in this thesis was a tomato oleoresin containing 6% lycopene by weight. Tomato oleoresin is a tomato extract containing many compounds other than lycopene, for this reason the possibility can not be excluded that the effects observed may be the result of some other substance or a synergistic effect with another substance. Lycopene made up greater than 90% of the total carotenoid profile therefore it is more likely that the observed beneficial effects are due to lycopene. Moreover, when comparing the effect of different lycopene preparations, including purified and oleoresin forms, on cellular proliferation of a human cancer cell line, all preparations showed a reduction in proliferation indicating that the protective property is acquired from lycopene rather than another component of tomatoes (Levy et al., 1995).

A concentration of 10 ppm lycopene was chosen because it represents a dietary dose of two servings of tomatoes or tomato products per day. In the Health Professionals Follow-up Study (Giovannucci et al., 1995), investigators found greater protection against the risk of prostate cancer in men consuming greater quintiles of tomato based products, with the highest protection offered to men who consumed more than ten servings per week. 10 ppm lycopene concentration is greater than eating ten servings of tomato products per week (it is equivalent to 14 servings per week) hence, was thought to be a sufficient concentration to notice differences.
Lycopene given at a dose of 10 ppm was found to be bioavailable and distribute to several tissues in the body. While the level of lycopene in animal tissues from different studies seems to be variable depending upon the amount of lycopene given, method of administration and duration of treatment of lycopene; there is agreement that lycopene tends to accumulate more so in the liver compared to other tissues (Lingen et al., 1959; Sharoni et al., 1997; Zhao et al., 1998). Most of the data looking at the accumulation of lycopene in tissues comes from human studies (Rao and Agarwal, 1999), and these values tend to be higher than lycopene levels from animal studies. It is difficult to compare feeding rats in the controlled conditions of animal studies with the multifaceted human situation. Consuming a variety of carotenoids together, as in eating a variety of fruits and vegetables, could be one reason for the higher levels of lycopene in human tissue. The bioavailability of lycopene was significantly greater when β-carotene and lycopene were given in combination compared to when lycopene was given alone (Johnson, 1998). Also, when lycopene from human tissue is reported in the literature, it is probably reflective of years of consumption which allows for more time for it to accumulate in tissues as opposed to short term feeding (two months in this study) in animal studies.

As mentioned earlier, lycopene is transported in blood via lipoproteins (Erdman et al., 1993; Parker, 1997). With the finding that lycopene was present in all tissues investigated indicates that there is effective transfer of lycopene from plasma lipoproteins to tissues in rats. The differential concentrations of lycopene in tissues suggests that there is likely a tissue specific mechanism which causes the selective uptake of lycopene. This type of
selective accumulation of carotenoids is also seen with lutein and zeaxanthin (Handelman et al., 1988; Bone et al., 1985). Both are found in high levels in the retina with zeaxanthin concentrated more so in the macula region. So far, no specific lycopene or carotenoid binding protein has been identified. Perhaps the selective tissue accumulation of lycopene is related to the activity of lipoprotein receptors, particularly LDL receptors. Finding high levels of lycopene in the prostate (Clinton et al., 1996) may explain why men who consume a greater amount of lycopene containing foods are at lower risk of developing prostate cancer (Giovannucci et al., 1995). After feeding 10 ppm dietary lycopene to rats; among all tissues assessed for lycopene, colon had one of the lowest lycopene level. At this dose, trends for a reduction in ACF incidence were observed. However, epidemiological data report that intake of tomatoes significantly decreased the risk of cancer of a number of digestive tract cancers including colon cancer (Franceschi et al., 1994). This indicates that the potential of lycopene to protect against colon cancer exists. Compared to the prostate, colon lycopene levels were approximately seven-fold lower. Therefore, if lycopene in the colon could be boosted to levels comparable to the prostate, then perhaps statistically significant findings in ACF incidence may be seen.

At this dietary dose, lycopene reduced biomarkers for in vivo oxidation. This reduction was significant for lipid peroxidation ($p < 0.02$) but not for protein oxidation. This observation is not unexpected. Since lycopene is a highly lipophilic molecule, it would be expected that lycopene would have a greater effect as an antioxidant in the lipid compartments of the body. The most widely present macromolecules in cells are lipids,
followed by proteins. Consequently, the more frequent target of free radicals would be lipids. Also, membrane proteins are less susceptible to oxidative damage compared to soluble proteins for the same reason (Nakazawa et al., 1996). This may explain why while there was a decrease in both lipid and protein oxidation, lipid peroxidation was significantly reduced. These results are also in agreement with the in vivo oxidation results from a human study where again, lipid peroxidation was significantly reduced while protein oxidation showed trends of decrease in all lycopene treatment cases (Rao and Agarwal, 1998a). It is also possible that there may actually be a significant difference in protein oxidation between control and treatment groups but that the methodology used to assay protein oxidation was not sensitive enough to pick up this difference. These animals were fed lycopene for a period of eight weeks, perhaps significance could have been reached if the feeding time was longer. The assay used to measure protein oxidation in this study measured reduced thiols of total serum proteins. Albumin is the most abundant protein in serum (Hu, 1994). Albumin also has a fast turnover rate. Protein oxidation assay of a more specific protein fraction could perhaps provide significant results.

At the dose of 10 ppm, while lycopene did not significantly reduce the incidence and size of colonic preneoplastic lesions (ACF), definite trends were observed. Both incidence and size of ACF were reduced in groups treated with lycopene and this inhibition was found to be maximal during the promotion phase of carcinogenesis and upon larger sized foci. Based on these results, it appears that lycopene does indeed possess some
anticarcinogenic capacity as seen by the potential to suppress ACF development. Furthermore, the decrease in protein oxidation and the significant reduction in lipid peroxidation indicates that lycopene is acting as an antioxidant. Therefore, the proposed sequence of events for these observations is illustrated in figure 9.1. Dietary lycopene raises lycopene in serum and tissues and reduces oxidation, thereby reducing the risk of cancer.

After performing a post hoc power analysis, it was calculated that 60 animals per test group would be required in order to observe statistically significant differences in ACF incidence. This is an impractical number to use in animal experiments. There could be a number of reasons why results of ACF incidence and size did not reach significance. Firstly, the concentration of lycopene used may not have been sufficiently high. As discussed in chapter 7, most other cancer studies using animal models used much higher levels of lycopene, as much as thirty times higher. With higher levels of administration, significant protective effects were seen against bladder, liver, mammary and lung carcinogenesis (Okajima et al., 1998; Astorg et al., 1997; Sharoni et al., 1997; Kim et al., 1997). If lycopene levels fed were too low, then this could possibly affect the amount of lycopene in serum. The majority of these studies did not look at serum lycopene levels, but one study (Sharoni et al., 1997), gave 10 mg lycopene per kg body weight twice weekly. Plasma lycopene levels were approximately 2 μg/ml. This value is 100 times greater than the serum lycopene levels from this study. Perhaps greater levels of lycopene in serum would cause a greater beneficial effect upon the organism. This may be achieved through administering a diet with a greater concentration of lycopene.
Figure 9.1. Proposed mechanism of the inhibitory effect of dietary lycopene on AOM induced colonic preneoplasia. Dashed lines indicate weaker inhibition, bold lines indicate stronger inhibition.
As proposed in figure 9.1, feeding rats a diet with higher levels of lycopene would give rise to higher lycopene in blood. With increased lycopene in blood, a greater amount of lycopene will be available to all body tissues, including the colon. As the colon was one of the tissues containing the lowest levels of lycopene, it is possible that the lack of statistical significance was because there may not have been sufficient lycopene present. However, a study which used lycopene at a lower dose than 10 ppm also found significant reduction in mammary tumor development in mice (Nagasawa et al., 1995). Therefore, the concentration of lycopene used may only be one factor affecting the outcome of preneoplasia.

Another reason, and maybe more pertinent one, could be the length of treatment. In this thesis the maximum time period rats were fed lycopene was fifteen weeks. Many other animal studies have looked at much longer periods than this and found significant protection against cancer risk. In a spontaneous mammary tumor model in mice, lycopene was fed for thirteen months (Nagasawa et al., 1995). Not only did mammary tumors develop two months later in lycopene treated animals but at any given time, the lycopene fed group was found to always have lower mammary tumor incidence compared to controls. In a carcinogen induced mammary tumor model, lycopene was administered to rats for 28 weeks (Sharoni et al., 1997). Investigators found that the number of tumors was lowest in the lycopene group, but the difference was obvious only at longer periods of time and was highly significant only at the last time point. Similarly, they found a trend for reduced tumor area with time, which was not significant. They monitored the growth
of tumor area for forty-three days. It is possible that if they continued monitoring after forty-three days, they would have reached significance. Furthermore, other studies which gave lycopene for shorter than fourteen weeks did not consistently show protection against carcinogenesis (Narisawa et al., 1996) or statistically significant results (Kim et al., 1998), further supporting the idea that length of time of administration is an important factor. In this thesis, progressively greater inhibition of larger foci size was observed which is analogous to lower that area occupied by preneoplastic lesions in lycopene fed animals. Perhaps, if the study continued to tumor stage, then results would have reached significance.

Another reason to believe that the study period may have been too short to notice statistically significant differences was the observation that with lycopene treatment during either the initiation or promotion phase, the greatest suppression of ACF incidence and size and also in foci of different aggressive states, was during the promotion phase. Promotion is the longer stage in carcinogenesis, indicating that long term exposure to lycopene gives maximal beneficial effect. Therefore, feeding lycopene for longer that fourteen weeks would perhaps amplify differences further.

Evidence from epidemiological studies provide further reason to think that time is an important factor. These studies have shown protective effect of dietary or serum lycopene is against risk for various cancers (Dorgan et al., 1998; Giovannucci et al., 1995; Franceschi et al., 1994; VanEewyck et al., 1991). Dietary and serum levels of carotenoids
are probably reflective of eating patterns and behavior of several years, in which case, protective effects are the result of chronic consumption of lycopene containing foods. Translating a lifetime consumption of lycopene containing products into a laboratory model for animals may require feeding experiments spanning more than 14 weeks.

Perhaps it may have been possible to show significant results in the short time period of 14 weeks if there was greater oxidative challenge to the organism and if a cell proliferative stimulus was provided. Humans are faced with a variety of exogenous sources of oxidants (cigarette smoke, environmental pollutants, ultraviolet rays, radiation, diet, etc) (Jacob and Burri, 1996; Dreher and Junod, 1996). The rats in this experiment may have already been in a low oxidative environment, in other words, the oxidative stress they experienced may have been very little. Little oxidative stress would result in only slight oxidative damage implying that only a small amount of protection from antioxidants would be required, too small to result in significance. This would explain that while lowered protein oxidation in serum and trends for reduced ACF incidence and size in rats on lycopene supplemented diet were observed, these results did not reach statistical significance.

This low level of oxidative tension may not be reflective of the real life situation in the case of human beings. As mentioned above, there are numerous external sources of oxidants of which some contribution is made by dietary elements such free fatty acids (Dreher and Junod, 1996). It is widely known that diets high in fat are associated with
several chronic diseases including cancer. The detrimental effects from fats is thought to occur through lipid peroxides, oxidation products of lipids (Dreher and Junod, 1996). Thus, it stands to reason that the more fat (free fatty acids) present in the diet, the more lipid peroxides will also be present, hence putting the individual at a higher risk for cancer development. If humans consume 30-40% of calories from fat, then the 4% of fat in the AIN93M rat diet represents a very low intake of fat, and accordingly a lower risk for cancer development. In addition, the fat source in the rat diet is soya oil, a healthy fat source. All of these point to a healthy basal diet for rats and not seemingly to pose any oxidative threat to the organism. Increasing the level of fat may not only lead to increased levels of lipid peroxides, thus oxidative burden, but also better reflect human dietary levels of fat.

Nonetheless, dietary lycopene shows definite trends for providing protection against in vivo oxidation and putative preneoplastic lesion growth. Although statistical significance was not seen, the biological significance of lycopene is evident. If, in future work it is found that lycopene indeed retards the growth of ACF, then the implication from this would be seen as the slowing of progression of tumors to malignancy.

Evidence from various mechanistic studies has found lycopene to provide important biological effects other than acting as an antioxidant. Lycopene was found to upregulate gap junctional communication by increasing the expression of the connexin 43 gene (Zhang et al., 1991). It may also enhance the immune system (Lingen et al., 1959).
Lycopene may also affect the cytochrome P450 activation system in the liver (Astorg et al., 1997). It was also found to be a powerful inhibitor of cellular proliferation of cancer cells (Levy et al., 1995). The anticarcinogenic activity of lycopene may partly be the outcome of the above mentioned properties.

In this thesis, lycopene was found to function both as an antioxidant as well as displaying some anticarcinogenic potential. It is possible that lycopene could be functioning by two independent mechanisms, one for reducing in vivo oxidation and another for suppressing the growth of ACF. Yet, it may also be possible that lycopene present in the target tissue, acts as an antioxidant to reduce oxidation and then this decrease in oxidation is translated to a reduction in cancer risk. Although the mechanism of action for lycopene is not fully understood, the effects are not a result of retinoic acid since lycopene is not a precursor for vitamin A. More research is required to elucidate the anticarcinogenic potential of lycopene and its underlying mechanism of action.

Colon cancer is a highly prevalent cancer and treatment usually fails. Since the cost of treatment of cancer is high, preventative strategies become important. Also, it is believed that up to 90% of colon cancer incidence variation is due to dietary choices. therefore, it has a high potential to be prevented (Pappalardo et al., 1996). Observations from this work indicate that the dietary intake of lycopene may provide protection against colon tumorigenesis. RDA recommendations for healthy eating include eating a minimum of five servings of fruits and vegetables daily. Two servings of lycopene containing fruits or
vegetables such as tomatoes should be considered as part of a daily regime in contributing to fulfilling these recommendations.

Research focus on lycopene is relatively recent. In addition to being a superior antioxidant, some other noteworthy aspects of lycopene are its wide occurrence in the human diet and its apparent absence of toxicity. If lycopene does indeed have beneficial properties against cancer development, the implications will be far reaching. Research on lycopene will deserve more priority. There will be greater involvement of agriculture community in growth of tomatoes and commercial companies in incorporating tomatoes in more food items.

9.2 Future investigations

The anticarcinogenic activity of lycopene has been suggested from various studies, however further work is needed in the area of its effect in colon cancer. Some projects of interest are listed below.

a) Conducting an animal study to see the effect of feeding higher lycopene levels (supplemental doses) to rats on ACF incidence.

b) Maintaining a dietary dose of lycopene and feed animals for a longer time period, and looking the effect on colonic tumor incidence.

c) Perform an assay to measure the effect of lycopene on cellular proliferation in colonic tissue, e.g. uptake of bromodeoxyuridine

d) Induce oxidative stress to animals, for instance, by feeding oxidized free fatty acids or
iron and observe the effect on preneoplasia.

e) Feeding specific lycopene isomers to colon cancer induced rats to see if there are differences in biological activities.

f) Conducting a randomized, double blind intervention trial in colon cancer patients.

g) Induce cell proliferation by incorporating cholic acid in the diet.
CHAPTER 10

REFERENCES


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APPENDIX A

AIN-93M Purified rodent diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams/kg</th>
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<td>Casein</td>
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<td>Cornstarch</td>
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<tr>
<td>DYETROSE</td>
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</tr>
<tr>
<td>Sucrose</td>
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</tr>
<tr>
<td>Cellulose</td>
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</tr>
<tr>
<td>Soybean oil</td>
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</tr>
<tr>
<td>Salt mix</td>
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</tr>
<tr>
<td>Vitamin mix</td>
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</tr>
<tr>
<td>L-Cystine</td>
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</tr>
<tr>
<td>Choline bitartrate</td>
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AIN-93M Mineral mix (35g/kg diet)

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<td>Calcium carbonate</td>
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<td>Potassium phosphate, monobasic</td>
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<tr>
<td>Potassium citrate . H2O</td>
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<td>Manganese carbonate</td>
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<td>Sucrose</td>
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### AIN-93-VX Vitamin mix (10g/kg diet)

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<td>Vitamin E acetate (500IU/g)</td>
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<td>Vitamin B12 (0.1%)</td>
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<tr>
<td>Vitamin D3 (400,000 IU/g)</td>
<td>0.25</td>
</tr>
<tr>
<td>Vitamin K1/Dextrose mix (10mg/g)</td>
<td>7.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>967.23</td>
</tr>
</tbody>
</table>

(Dyets Inc., Bethlehem, Pennsylvania)
APPENDIX B

Tomato oleoresin composition

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>CONTENT %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acids as glycerides</td>
<td>71.4±1.9</td>
</tr>
<tr>
<td>Total unsaponifiable matter</td>
<td>16.4±2.1</td>
</tr>
<tr>
<td>Lycopene</td>
<td>5.7±0.7</td>
</tr>
<tr>
<td>Phytoene</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>0.6±0.3</td>
</tr>
<tr>
<td>Tocopherol</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>Sterol</td>
<td>1.9±0.5</td>
</tr>
<tr>
<td>Water soluble matter</td>
<td>3.6±0.8</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.58±0.1</td>
</tr>
<tr>
<td>Water content</td>
<td>0.61±0.2</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.43±0.1</td>
</tr>
<tr>
<td>Phospholipids (estimated from phosphorus)</td>
<td>11.5±2.1</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>0.21±0.1</td>
</tr>
<tr>
<td>Ash</td>
<td>0.74±0.1</td>
</tr>
</tbody>
</table>

Fatty acid profile

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>% of total peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid (14:0)</td>
<td>0.52±0.02</td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>22.65±0.27</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>5.26±0.09</td>
</tr>
<tr>
<td>Oleic acid (18:1)</td>
<td>12.91±0.41</td>
</tr>
<tr>
<td>Linoleic acid (18:2)</td>
<td>47.96±0.84</td>
</tr>
<tr>
<td>Linolenic acid (18:3)</td>
<td>9.65±0.98</td>
</tr>
<tr>
<td>Arachidic acid (20:0)</td>
<td>0.97±0.06</td>
</tr>
<tr>
<td>Behenic acid (22:0)</td>
<td>0.52</td>
</tr>
</tbody>
</table>

(LycoRed Natural Products Industries Ltd.)
### Sterol contents

<table>
<thead>
<tr>
<th>Sterol</th>
<th>% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stigmasterol</td>
<td>0.62±0.15</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>0.54±0.10</td>
</tr>
<tr>
<td>Campesterol</td>
<td>0.54±0.15</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.23±0.07</td>
</tr>
</tbody>
</table>

### Carotenoids in tomato oleoresin

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>% of total OD at 472nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lycopene</td>
<td>96.5±0.6</td>
</tr>
<tr>
<td>β-carotene</td>
<td>1.8±0.5</td>
</tr>
<tr>
<td>Oxidized products</td>
<td>1.8±0.2</td>
</tr>
</tbody>
</table>

### Tocopherol, phytoene and phytofluene contents

<table>
<thead>
<tr>
<th>Compound</th>
<th>% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoene</td>
<td>1.02±0.24</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>0.62±0.29</td>
</tr>
<tr>
<td>tocopherols</td>
<td>0.4±0.1</td>
</tr>
</tbody>
</table>

(LycoRed Natural Products Industries Ltd.)