REDUCING THE RISK OF PROSTATE CANCER WITH TOMATO AND SOY BIOACTIVES

BY

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DISSERTATION

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ABSTRACT

Prostate cancer (PCa) is the second leading cause of cancer and cancer-related deaths in U.S. men. Dietary strategies to prevent or reduce PCa would have a significant impact on public health. The American Institute for Cancer Research (AICR) recommends consuming a variety of fruits, vegetables, whole grains, and legumes for cancer prevention. Specifically, epidemiological studies suggest that consumption of tomato or soy products may reduce the risk of PCa.

Tomatoes contain many bioactive components, but the tomato carotenoids, lycopene, β-carotene, phytoene, and phytofluene, receive much of the attention regarding PCa incidence. As tomato carotenoids are lipophilic, strategies to enhance absorption and tissue distribution have been of interest. Evidence suggests that co-consumption of tomato carotenoids with a source of dietary fat increases their absorption and bioavailability, however, the influence of fat type on tomato carotenoid tissue accumulation has not been well understood. Therefore, we fed Mongolian gerbils (Meriones unguiculatus) a 20% fat diet containing 10% tomato powder (TP) and either safflower oil (polyunsaturated fat) or coconut oil (saturated fat) and determined the influence of dietary fat type on tissue carotenoid bioaccumulation.

Coconut oil fed-gerbils had increased tissue carotenoid concentrations including total carotenoids in the serum (p=0.0003), adrenal glandular phytoene (p=0.04), hepatic phytofluene (p=0.0001), testicular all-trans lycopene (p=0.01), and cis-lycopene (p=0.006) in the prostate-seminal vesicle complex compared to safflower oil. Safflower oil-fed gerbils had greater splenic lycopene concentrations (p=0.006) compared to coconut oil-fed gerbils. Additionally, coconut oil feeding increased serum cholesterol (p=0.0001), and decreased hepatic cholesterol (p=0.0003) compared to safflower oil. Coconut oil enhanced tissue uptake of tomato carotenoids to a greater
degree than safflower oil, and these results may have been due to the large proportion of medium chain fatty acids in coconut oil which might have caused a shift in cholesterol flux to favor extrahepatic carotenoid tissue deposition.

*In vivo* studies examining the effect of TP or tomato paste on PCa have demonstrated mostly positive results. However, these studies often model lifelong consumption of tomato products (initiated post-weaning, ~4 weeks of age) and may not accurately reflect the changes in dietary patterns or supplement use in men newly diagnosed with PCa. Therefore, we designed a 10% TP diet intervention in TRAMP mice to determine the effects of consuming TP (post-puberty, ~5-6 weeks of age) during the process of carcinogenesis on PCa incidence.

8-week-old male C57BL/6 X FVB TRAMP mice were randomized to consume either an AIN-93G + 10% TP diet (N=90) or the AIN-93G control diet (N=88) and assigned to one of three sacrifice ages: 12 (N=59), 16 (N=60), or 20 (N=59) weeks. Overall cancer incidence was not impacted by diet at any time point. However, at 16 weeks of age, TP significantly increased high-grade PIN (HGP) (p=0.014) and significantly decreased poorly-differentiated (PD) (p=0.024) lesions compared to the control diet suggesting a modest reduction in cancer progression by TP at 16 weeks of age.

There are two variables that may explain the modest effect of TP in this study: the low amount of lycopene in the TP diet and the timing of the dietary intervention. The TP diet contained 30-fold less lycopene than a previous study in our lab. However, after 8 and 12 weeks of feeding, mice accumulated similar tissue concentrations of carotenoids in their serum, gonadal adipose, and prostate suggesting that the amount of carotenoids in the diet may not have been the critical factor since tissue levels of lycopene appear to become saturated with long-term feeding.
Rather, the initiation of the diet intervention at 8 weeks of age instead of 4 weeks of age may have been too late in cancer development to substantially impact carcinogenesis, as studies have shown prostate-specific gene expression changes as early as 8-10 weeks of age in TRAMP mice. We histopathologically confirmed that mice had low-grade and moderate-grade PIN at 8 weeks of age, when the dietary intervention was initiated. The TRAMP model is an aggressive model of PCa, therefore, diet may not be able to have as great of an impact when initiated after puberty (~6 weeks of age) when PCa is developing rapidly in this model. Both the late intervention time combined with low levels of carotenoids in the TP diet may have contributed to the lack of an overall effect. This study re-emphasizes the importance of lifelong consumption of TP for PCa prevention.

Epidemiological studies have also associated soy consumption with a decreased risk of PCa. One hypothesis for the decreased risk of PCa by soy consumption is the production of the microbial-metabolite, equol, from daidzein. A TRAMP study from our lab suggested that a 2% soy germ diet reduced PCa incidence by 34%. The same study observed that prostatic equol concentrations were 39 times greater than prostatic genistein and 3 times higher than prostatic daidzein suggesting that equol may have been a significant factor in reducing PCa incidence. We aimed to determine if equol was responsible for the protective effect of soy germ by replicating that study with additional dietary groups.

3-week old male C57BL/6 X FVB TRAMP mice were weaned from our breeding colony and immediately acclimated to an AIN-93G control diet for one week. At 4 weeks of age, mice (n=30 per diet group) were randomized to one of four pelleted study diets, AIN-93G control, AIN-93G + 2% soy germ, AIN-93G + 92 ppm daidzein, or AIN-93G + 88 ppm equol until 18 weeks of age. To our surprise, we did not detect any statistical differences in cancer incidence
between diets. In the previous study, we observed a 100% cancer incidence in TRAMP mice fed a control diet until 18 weeks of age, however, the control group in this study only had a 24% incidence in cancer at 18 weeks of age. Additionally, mice in the current study had less food intake and significantly decreased body weights (p<0.001) compared to those in the previous study. A reduction in food intake and body weight is known to reduce cancer incidence in a number of animal models and is likely to have contributed to the decrease in expected cancer incidence in the current study. We hypothesize that the reason for the decreased food intake, the decreased body weight, and decreased cancer incidence was due to the physical format of the study diets. Mice in the current study were fed pelleted diets, whereas mice in the previous study were fed powdered diets. Differences between powdered and pelleted diets exist and have been shown to influence obesity which, in turn, can affect cancer development.

Additionally, we measured serum levels of cytokines in mice with tumors and in mice with high-grade PIN. In mice with tumors, daidzein and equol diets significantly altered the levels of IL-1β, IL-10, IFN-γ, and TNF-α compared to the control and soy germ groups suggesting that daidzein and equol may play a role in moderating systemic inflammation in advanced PCa. While our diets did not impact overall cancer incidence in this study, the role of daidzein and equol in PCa still warrants investigation.

Overall our findings suggest that consuming fruits and vegetables containing bioactives such as carotenoids or isoflavones may be beneficial in reducing PCa incidence. The co-consumption of fat with carotenoids can increase their bioavailability and tissue biodistribution. Lifelong consumption of carotenoid-containing fruits and vegetables is likely more protective than changes to the diet or supplement use at the time of cancer diagnosis. Lastly, the format of the diet is important to consider when studying the effects of bioactives in cancer models.
Nonetheless, dietary strategies to reduce the risk of PCa remain of interest and of public health significance.
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CHAPTER 1

Literature Review

INTRODUCTION

Cancer development is a complex and multi-step process. In 2000, Hanahan and Weinberg outlined six essential alterations in cellular physiology which determine malignant growth\(^1\). Sustained proliferative signaling, evasion of growth suppressors, active invasion and metastases, acquired unlimited replicative potential, induced angiogenesis, and evasion of cell death were termed the Hallmarks of Cancer\(^1\). These six hallmarks were expanded in 2011 to include emerging hallmarks and enabling characteristics such as the avoidance of immune system destruction, tumor-promoting inflammation, genomic instability, and deregulated cellular energetics in an effort to further describe strategies cancer cells possess and develop to survive.

Once thought to be primarily influenced by hereditary factors alone, research now suggests only 5-10% of cancer cases can be attributed solely to hereditary causes\(^2,3\). The remaining 90-95% of cancer cases are thought to be primarily influenced by environmental factors including epigenetics and dietary habits\(^2\). The 2007 AICR/WCRF Report suggests that “cancer is 30-40% preventable over time by appropriate food and nutrition, regular physical activity, and avoidance of obesity”\(^4\). Though significant, the risk for cancer development depends on a multitude of factors including the entire diet profile, type of cancer, other environmental factors, and genetics of the individual. Despite evidence that suggests that the association between intakes of total fruits and vegetables and cancer incidences is weak, specific types of fruits and vegetables have been inversely associated with cancer incidences, including soy and tomatoes\(^5,6\).

Prostate cancer (PCa) is the most common cancer in men and is the second leading cause of cancer deaths in men in the US with an estimated 233,000 new cases diagnosed in 2014\(^7\). Risk
factors for PCa include age, genetics, family history, race, smoking, obesity, and diet\textsuperscript{8}. The pathological progression of PCa is classified as follows: normal prostate epithelium, prostatic epithelial neoplasia (PIN), localized PCa, metastatic PCa, and castration-resistant PCa (CRPC)\textsuperscript{9}. Due to increased screening and earlier detection of PCa, recommendations for localized prostate cancers often include “watchful waiting” or “active surveillance” which involves frequent monitoring of the disease by digital rectal examinations and prostate-specific antigen (PSA) levels in an attempt to avoid costly and unnecessary treatments with negative side effects. The slow-progressing nature of PCa combined with early detection provides an ideal window of time where dietary interventions may be beneficial. Men diagnosed with PCa often turn to dietary supplements as an inexpensive way to reduce the risk of cancer progression\textsuperscript{10}. The use of supplements marketed towards men for prostate health has been estimated at 13\% in men with PCa, but may actually be higher due to the unreported use of complementary and alternative medicine (CAM)\textsuperscript{10}. In the 2007 report, the American Institute for Cancer Research and World Cancer Research Fund (AICR/WCRF) emphasized a diet rich in plant foods to reduce cancer risk\textsuperscript{4}. There is a need to investigate the anticarcinogenic properties of bioactive compounds from fruits and vegetables for PCa protection.

\textbf{Tomato for Prevention of Prostate Cancer}

Tomato consumption has been linked to decreased risk of chronic diseases\textsuperscript{11}. In addition to vitamins A, C, and E, tomatoes are excellent sources of potassium, fiber, and folate\textsuperscript{12}. Furthermore, tomatoes contain a variety of flavonols such as quercetin and kaempferol and carotenoids such as lycopene, phytoene, phytofluene, α-carotene, ζ-carotene, and β-carotene\textsuperscript{12}. Individually, many components in tomatoes have been investigated for their anti-cancer and
antioxidant properties, but the combination of these components may best contribute to the numerous health benefits of tomatoes.

*Tomatoes and Prostate Cancer – Epidemiologic Evidence*

In 1995, Giovannucci et al. reported that the intake of lycopene-rich foods including tomatoes, tomato sauce, and pizza was associated with a significant risk reduction for total PCa (RR=0.65) and advanced PCa (RR=0.47). Recently, further analyses of data from the Health Professionals Follow-Up Study demonstrated a significant inverse association between lycopene intake and the incidence of lethal PCa (HR=0.72). These studies also suggest that early and/or lifelong lycopene intake is more important for PCa risk reduction than recent lycopene intake. A 2004 meta-analysis of 11 case-control studies and 10 cohort studies indicated a relative risk of 0.89 in men consuming high intakes of tomato products and PCa incidence, and a further reduction in relative risk (RR=0.81) in men consuming high intakes of cooked tomato products. The results of this meta-analysis suggest that processing tomatoes enhances their protective effect against PCa. In contrast, the results of the Prostate Cancer Prevention Trial and Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial did not observe a significant association between tomato products or lycopene, and PCa risk. The mixed results from the epidemiology were addressed in the 2014 Continuous Update Report for PCa from AICR/WCRF. In this updated report, evidence for lycopene’s effect on PCa was down-graded from “probable” to “limited-no conclusion” based on the analyses of recent human studies. While the classification of lycopene has changed, the report recognized that the nuances between different prostate cancers, study designs, and populations, complicates the interpretation of the evidence for broad classifications of food and PCa risk.
Tomatoes and Prostate Cancer - In vitro Evidence

The mechanism by which tomatoes and tomato products may reduce PCa risk has not been elucidated. The major carotenoid found in tomatoes, lycopene, is responsible for their red color and has been suggested as the primary bioactive constituent. Lycopene has been reported to protect against oxidation, induce cell-cycle arrest, inhibit proliferation, increase apoptosis, inhibit angiogenesis, and disrupt growth factor and hormonal signaling in vitro\textsuperscript{18–21}. One study demonstrated that lycopene combined with a common carbohydrate derivative found in dried tomato products, ketosamine, reduced growth in MAT-LyLu cells\textsuperscript{22}. That study suggested that other components in tomatoes may also be protective against PCa.

Tomatoes and Prostate Cancer – Pre-clinical Animal Trials

There are a handful of studies examining the effect of tomato powder (TP) on PCa in vivo (Table 1.1)\textsuperscript{20,23–25}. In N-methyl-N-nitrosourea (NMU) testosterone-treated rats, a 10% TP diet increased overall PCa-free survival compared to the control diet and the lycopene supplemented diet\textsuperscript{23}. The findings from this study suggested that components in TP, in addition to lycopene, were protective against PCa development. In the Dunning R3327-H model of PCa, a 10% TP diet significantly decreased tumor weights, increased apoptosis, and decreased proliferation compared to the control diet\textsuperscript{20}. Furthermore, lycopene alone did not significantly reduce tumorigenesis, further supporting the hypothesis that tomatoes contain other bioactive compounds necessary for PCa prevention. In the transgenic adenocarcinoma of the mouse prostate (TRAMP) PCa model, a 10% TP diet increased survival and decreased PCa incidence compared to the control diet\textsuperscript{24,26}. Interestingly, it appears that TP may be more effective than tomato paste in animal studies of PCa. When PCa incidence was compared in the TRAMP model between diets matched for lycopene from tomato paste or supplemented from a commercial lycopene beadlet
source, only the lycopene beadlet diet significantly reduced PCa incidence compared to the control\textsuperscript{27}. TP is manufactured with the tomato seeds and skins included, whereas tomato paste is processed without the seeds and skin\textsuperscript{27}. It may be that compounds found in tomato seeds and/or skin may be protective against PCa. Overall, the vast majority of pre-clinical animal trials support the evidence from epidemiology that whole tomato products are more effective than lycopene alone at reducing the risk for PCa.

Table 1.1. Summary of Rodent Studies of 10\% Tomato Powder and Tomato Paste Diets and PCa Risk

<table>
<thead>
<tr>
<th>Author</th>
<th>Animal Model</th>
<th>Dietary Source of Lycopene</th>
<th>Lycopene Exposure</th>
<th>Study Outcome</th>
<th>Effective?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zuniga et al. 2013\textsuperscript{24}</td>
<td>TRAMP mice</td>
<td>10% Tomato Powder</td>
<td>286 ppm</td>
<td>PCa Incidence</td>
<td>Yes</td>
</tr>
<tr>
<td>Pannelini et al. 2010\textsuperscript{28}</td>
<td>TRAMP mice</td>
<td>10% Tomato Powder</td>
<td>132 ppm</td>
<td>Survival</td>
<td>Yes</td>
</tr>
<tr>
<td>Konijeti et al. 2010\textsuperscript{27}</td>
<td>TRAMP mice</td>
<td>Tomato Paste</td>
<td>28 ppm</td>
<td>PCa Incidence</td>
<td>No</td>
</tr>
<tr>
<td>Wan et al. 2014\textsuperscript{29}</td>
<td>TRAMP mice</td>
<td>10% Tomato Powder</td>
<td>384 ppm</td>
<td>Androgen Metabolism and Signaling</td>
<td>Yes</td>
</tr>
<tr>
<td>Boileau et al. 2003\textsuperscript{23}</td>
<td>(NMU)-Testosterone- treated rats</td>
<td>10% Tomato Powder</td>
<td>13 ppm</td>
<td>PCa Risk</td>
<td>Yes</td>
</tr>
<tr>
<td>Mossine et al. 2008\textsuperscript{22}</td>
<td>(NMU)-Testosterone- treated rats</td>
<td>10% Tomato Powder</td>
<td>unknown</td>
<td>Survival</td>
<td>Yes</td>
</tr>
<tr>
<td>Canene-Adams et al. 2007\textsuperscript{20}</td>
<td>Dunning R3327-H rats</td>
<td>10% Tomato Powder</td>
<td>7 ppm</td>
<td>Prostate Tumor Weight</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Tomatoes and Prostate Cancer – Clinical Trials

Several clinical trials have investigated the effect of lycopene supplementation and tomato product consumption in men with PCa. Intervention studies have shown that consumption of tomatoes or tomato products may protect against DNA strand breaks induced by
reactive oxygen species (ROS)\textsuperscript{30}. Only a handful of randomized controlled clinical trials have tested lycopene’s efficacy in humans with PCa. Kucuk et al. determined that patients consuming 15 mg of lycopene a day for 3 weeks prior to prostatectomy had decreased serum PSA levels and increased microscopically free resection margins compared to the control\textsuperscript{31}. A 2 year intervention of 2 mg of lycopene twice a day significantly reduced serum PSA and significantly increased survival rate in men with metastatic PCa compared to the control\textsuperscript{32}. In contrast, 15 mg of lycopene twice a day for 6 months did not reduced serum PSA compared to the control group\textsuperscript{33}. And an 8 week intervention of lycopene and soy protein resulted in no changes in serum PSA between the groups\textsuperscript{34}. Although human clinical trials have not yet demonstrated the chemopreventative effects of lycopene that pre-clinical rodent studies and epidemiological studies have shown, there is a need for additional well-designed and controlled intervention studies in men with PCa.

3-Dimensional (3D) Ultrasound Imaging and Prostate Cancer

Currently, 3-D ultrasound imaging is routinely used to guide systematic biopsy for PCa diagnosis and to monitor treatment response. The development of high-resolution ultrasound imaging for small laboratory animals provides a novel method to monitor tumor characteristics and changes in preclinical models. 3-D imaging has been able to detect tumor burden in two transgenic PCa models\textsuperscript{35}. The use of 3-D longitudinal imaging to monitor dietary intervention provides a non-invasive means to quantify tumor burden and metastasis \textit{in vivo}.

Soy and Prostate Cancer

Epidemiological studies suggest a reduced risk of PCa in men who consume soy products\textsuperscript{36}. Soy products contain an array of bioactive compounds including saponins, lignans, and isoflavones, yet much of the research in cancer prevention has focused on the predominant
isoflavone found in soy foods, genistein. Genistein has been investigated for its anti-proliferative, antioxidant, and chemopreventive properties in cell, animal, and human models of PCa\textsuperscript{37}.

\textit{Soy and Prostate Cancer – Epidemiologic Evidence}

A disparity exists between PCa incidences around the world. The PCa incidence in Japan is one-fifth of that in the United States\textsuperscript{38}. Migration studies have shown that PCa risk increases in men migrating from areas of low incidence to areas with high incidence\textsuperscript{38}. Lifestyle, environmental, and dietary factors have been widely hypothesized to contribute to this variation. In addition to low intakes of saturated fat and higher intakes of dietary fiber, Asian populations also consume more soy products\textsuperscript{39}. Mean serum isoflavone concentrations are higher in men from Japan than men from the United Kingdom and Finland suggesting that soy consumption may be responsible for differential PCa risk in these populations\textsuperscript{38}. In the Japan Public Health Center (JPHC) – based Prospective Cohort Study, consumption of soy products, genistein, and daidzein were associated with a decreased risk of localized PCa\textsuperscript{38}. A 2009 meta-analysis of 6 cohort and 8 case-control studies, described a decreased risk of PCa (RR/OR of 0.74) with total soy consumption\textsuperscript{40}. The RR/OR for Asian populations was 0.52 and for studies in Western populations was 0.99\textsuperscript{40}. The dietary differences between populations with differing incidences of PCa may provide insight into mechanisms by which soy may reduce PCa risk.

\textit{Soy and Prostate Cancer – In vitro Evidence}

The mechanism by which genistein and soy isoflavones protect against PCa has been extensively investigated \textit{in vitro}. Soy isoflavones have structural similarity to 17β-estradiol and have weak estrogenic activity\textsuperscript{41}. It’s hypothesized that phytoestrogens, such as isoflavones, work primarily through estrogen receptor β (ERβ), though the exact mechanism is unknown. ERβ,
which has been shown to be anti-proliferative, has been proposed to be an important driver of prostatic epithelial differentiation\textsuperscript{42}. The use of ERβ-agonists in mice with prostatic hyperplasia resulted in a reduction of cellular proliferation and an increase in apoptosis in the prostate, and ERβ is suggested to be a promising target for PCa treatment\textsuperscript{42,43}. Androgen receptor (AR) is a well-known regulator of benign and malignant prostatic proliferation and differentiation and is a the common target of current PCa therapeutic treatment\textsuperscript{42}, however, the use of anti-androgens to bind AR can lead to a PCa that is incurable. \textit{In vitro} studies have shown isoflavones induce cell-cycle arrest, reduce cancer cell proliferation, and modulate cell-cycle signaling\textsuperscript{41}. While genistein has been associated with a protective effect against several cancers and diseases, further research is needed to elucidate the effects of the other soy isoflavones and their metabolites, including glycinein, daidzein, dihydrodaidzein, and equol, and their impact on human health and disease prevention.

\textit{Soy and Prostate Cancer – Pre-clinical Animal Trials}

Isoflavones, most notably genistein, have long been investigated for their effects in animal models of PCa. In Sprague-Dawley rats, 250-1000 ppm dietary genistein per day reduced AR and ER expression in the prostate\textsuperscript{44,45}. Dietary genistein also reduced the incidence of poorly differentiated (PD) lesions in TRAMP mice in a dose-dependent manner\textsuperscript{37}. In addition to genistein, studies using mixtures of isoflavones or isoflavones from soy products have also been effective at reducing PCa. Lobund-Wistar rats fed an isoflavone-rich diet had a significant reduction in tumor growth compared to control rats\textsuperscript{46}. Soy germ is a commercially available supplement containing 50\% of isoflavones from daidzein, 39\% from glycinein, and 11\% from genistein. It was recently demonstrated by our laboratory to decrease the incidence of PD PCa in the TRAMP model by 34\% compared to the control\textsuperscript{24}. Lastly, a diet containing soy protein
reduced tumor weight and volume in nude mice transplanted with LNCaP cells compared to the control diet\textsuperscript{47}. Despite the growing body of evidence that soy products are protective against PCa, the mechanisms of protection by a combination of isoflavones and/or metabolites are relatively unknown.

\textit{Soy and Prostate Cancer - Clinical Trials}

Clinical trials using soy products have yielded mixed results concerning PCa prevention. In a clinical trial of 38 PCa patients, consumption of 160 mg/day of isoflavones from red clover resulted in a significant increase in apoptosis in prostate tumors\textsuperscript{48}. Furthermore, in men with PCa, consumption of soy or isoflavones significantly decreased serum PSA compared to the controls\textsuperscript{49-51}. This effect, however, was not observed among all studies in this population\textsuperscript{48,52}. Lastly, the results of a phase II clinical trial in men with PCa given isoflavone supplements containing 52 mg of total isoflavone aglycone equivalents observed no change in PSA\textsuperscript{53}. The conflicting results concerning PCa may be attributed to dose administered, sources of isoflavones, bioavailability of isoflavones, or specific type of soy intervention. Furthermore, differences between individuals including cancer stage and their ability to produce equol may further confound results.

\textit{Equol and Prostate Cancer}

Epidemiological studies suggest an association between isoflavone intake and a decreased risk of PCa, especially in men who produce equol\textsuperscript{36}. Equol is produced by the microbial metabolism of daidzein in the large intestine. It’s estimated that only 25-30\% of the populations in Western countries are equol “producers” compared to up to 80\% of individuals in China, Japan, and South Korea\textsuperscript{54,55}. An individual’s ability to produce equol is not fully understood, but it is thought that the gut microbiome, host genetics, and diet appear to contribute
to equol production from daidzein\textsuperscript{56}. If the benefits from soy consumption are dependent upon equol production, an estimated 70-75\% of the Western population would not benefit from soy consumption. Therefore, research is needed to determine whether cancer protection by soy consumption is dependent on equol production. Habitual intake of soy products early in life may be required to become an equol producer, as consumption of isoflavones in adulthood does not always convert a non-producer of equol into a producer\textsuperscript{55}. Previously we observed that prostatic equol was 39 times higher than genistein and 3 times higher than daidzein in mice consuming soy germ\textsuperscript{24}. The elevated concentration of equol in the prostate suggests that equol was a potential major contributor to the anticarcinogenic effects of soy germ observed in the TRAMP model\textsuperscript{24}.

Equol has been demonstrated to be more estrogenic than daidzein\textsuperscript{54} and has a higher binding affinity for ER\textbeta. Equol has been shown to bind ER\alpha and ER\textbeta with similar affinity as genistein\textsuperscript{57}. Equol has also been shown to bind 5-\alpha-dihydroxytestosterone (DHT) \textit{in vivo} thereby reducing prostate growth by sequestering DHT from the AR\textsuperscript{58}. By sequestering DHT from the AR, equol functions similar to anti-androgen drugs without interfering with DHT synthesis or AR signaling, which can have additional biological consequences. Equol was also shown to inhibit PCa cell growth and invasion by down-regulating matrix metalloproteinases \textit{in vitro}\textsuperscript{59,60}. Equol’s ability to be a selective estrogen modulator as well as an androgen antagonist makes it a promising target to study for hormonally-driven cancers like PCa. Limited \textit{in vivo} studies have directly examined feeding equol and the incidence of PCa in rodents. Due to the previously observed protective effect of soy germ from our lab\textsuperscript{24}, additional research is needed to elucidate if the protective effect of soy germ is attributed to equol production and if so, if the protective effect of soy germ limited to equol producers.
**Tomato Carotenoid Bioavailability and Absorption**

The structure of carotenoids is based on a C40 isoprenoid backbone that may be cyclic or acyclic and have polar groups. Carotenoids containing at least one oxygen atom are classified as xanthophylls, while carotenes are characterized by a hydrocarbon structure. While over 600 carotenoids have been identified in nature, only 60 or so appear to be consumed in the diet. Interest in the structure and function of carotenoids stems from epidemiological evidence supporting the protective effects of carotenoid-rich fruits and vegetables against many chronic and degenerative diseases including cardiovascular disease, age-related macular degeneration, and some cancers.$^{14,61,62}$

Bioavailability describes the degree to which ingested compounds like carotenoids may be absorbed and utilized from various food sources.$^{63}$ Once released from the food matrix, carotenoids are incorporated into mixed micelles with bile salts where they are then believed to be transported into intestinal cells through the B type-1 (SRB-1) receptor as well as the cluster determinant 36 (CD36)$^{64,65}$. Once inside intestinal cells, carotenoids are packed into chylomicrons and secreted into the lymphatic system$^{64}$. In the plasma, carotenoids are transported via lipoproteins and distributed to different tissues. There are several factors that influence carotenoid absorption and bioavailability including carotenoid species, the food matrix, processing factors, host genetics, nutrient interactions, and the co-consumption of dietary fat.$^{63,66}$ The presence of dietary fat with a carotenoid-containing meal has been shown to increase carotenoid absorption and bioavailability in humans.$^{67-69}$ Interestingly, the type of dietary fat has also been shown to impact carotenoid bioavailability. In humans, beef tallow (rich in saturated fat) increased post-prandial $\beta$-carotene content in chylomicrons compared to sunflower oil (polyunsaturated-rich fat)$^{70}$. However, butter reduced the post-prandial chylomicron response...
when compared to monounsaturated olive oil and polyunsaturated sunflower oil. In addition to degree of saturation, fatty acid chain length also appears to be an important aspect of carotenoid absorption and bioavailability. Medium chain triglycerides appear to be better at solubilizing carotenes, whereas long-chain triglycerides may facilitate micellarization and incorporation into chylomicrons. Differing structural properties and degrees of saturation appear to play a key role in carotenoid absorption and metabolism.

Upon distribution to tissues, carotenoids such as β-carotene, α-carotene, and β-cryptoxanthin are cleaved symmetrically at their central double bond by β-carotene 15, 15'-oxygenase (BCO1). β-carotene can be cleaved into two molecules of retinal which can be further reduced to retinol or oxidized to retinoic acid. BCO1 has been shown to have little or no activity cleaving non-provitamin A carotenoids in vivo. The existence of β-carotene-9, 10'-oxygenase (BCO2) has been detected in mice, humans, and zebra fish and shares an overall sequence homology with BCO1. BCO2 has a stronger affinity for cleavage of non-provitamin A carotenoids, such as lycopene, lutein, and zeaxanthin. BCO2 cleaves eccentrically resulting in apo-carotenals. The biological effects of apo-lycopenals are largely unknown, but apo-lycopenals have been shown to be metabolically active and may play important roles in the protective effect of tomatoes.

Soy Isoflavone Absorption and Bioavailability

Isoflavones are present in soy-containing foods as β-glycosides, aglycones, malonyl-glycosides, and acetyl-glycosides. Upon digestion of soy products, β-glucosidases located on the brush border membrane of the intestine or from bacteria, cleave genistin, daidzin, and glycitin into a sugar and respective aglycone. The resulting aglycones, genistein, daidzein, and glycinein, are then absorbed by passive diffusion into the enterocytes, glycosylated,
glucoronidated, or sulphated by intracellular enzymes, and exit the enterocyte by facilitated transport into portal circulation. Aglycones that enter portal circulation can be conjugated in the liver, kidney, and other organs. Not all aglycones are absorbed by enterocytes. The ones that make it to the large intestine are metabolized by microbes. Genistein can be metabolized into dihydrogenistein and further reduced to p-ethyl phenol or 6’-hydroxy-o-desmethylangolensin. Little is known about the metabolites of glycitein. Demethylated glycitein metabolites have been detected in vitro and in vivo. Daidzein can be reduced into dihydrodaidzein and further metabolized into (S)-equol and O-desmethylangolensin (O-DMA).

**Safety and Efficacy of Soy Germ**

The vast majority of research on PCa and soy is focused on studies examining intake of soy foods (fermented and non-fermented) and isoflavone supplements. However, there are commercially-available alternative soy products that contain isoflavones that are marketed towards consumers for health benefits. Soy germ, the hypocotyl of the soy bean, is a commercially-available product derived from soy beans that is currently being investigated for its effects in inflammatory bowel disease (IBD), diabetes, and PCa. Soy germ is unique in that it is not chemically processed, but has a high concentration of isoflavones. The radically different isoflavone profile has been suggested to confer health benefits in humans. Soy germ contains a greater proportion of the isoflavone, daidzein, followed by glycitein, and then genistein. The larger proportion of daidzein may be significant because of the microbial conversion of daidzein into equol and the potential health benefits associated with equol. Soy germ-enriched pasta was shown to reduce serum LDL cholesterol, decrease arterial stiffness, reduce blood pressure, and reduce markers of oxidative stress in humans. Furthermore, the effects were more pronounced in individuals who were capable of producing equol, further
suggesting that equol production may be the key to the efficacy of soy germ. The incorporation of soy germ into a beverage for human consumption resulted in no toxicity, excellent compliance, and good absorption and bioavailability of isoflavones making it a suitable vehicle for future cancer prevention trials. In TRAMP mice fed a modified diet containing 2% soy germ, a 34% reduction in PCa incidence was observed. The results of these studies suggest that soy germ may be successfully incorporated into a variety of foods for disease prevention studies.

**The TRAMP model**

The TRAMP model is a transgenic model of PCa. The rat probasin promoter’s response to androgens at puberty causes the prostate-specific expression of the Simian virus 40 large T antigen which suppresses tumor suppressors, p53 and Retinoblastoma (Rb). The suppression of these genes results in uncontrolled prostatic proliferation and eventual carcinoma. The TRAMP model develops multi-stage prostate carcinogenesis that exhibits similar pathological and molecular features to human PCa starting with prostatic intraepithelial neoplasia (PIN) to well-differentiated carcinoma and eventually a poorly differentiated carcinoma with metastases primarily to the periaortic lymph nodes, liver, and lungs (Figure 1.1). Figure 1.2 shows examples of the range of histological grades of TRAMP mouse prostate tissue at 18 weeks of age. The TRAMP model has been extensively utilized to examine dietary bioactives’ anticarcinogenic properties.
Figure 1.1. Prostate Cancer Progression in the TRAMP Model\textsuperscript{87}

![TRAMP Model Diagram]

Figure 1.2. TRAMP Mouse Prostate Pathology Grades at 18 Weeks of Age

![Pathology Images]

Low-grade PIN (A), moderate-grade PIN (B), high-grade PIN (C), well-differentiated carcinoma (D), moderately-differentiated carcinoma (E), poorly-differentiated carcinoma (F). Images were captured from Nanozoomer-scanned slides with NDP view software at 40X digital zoom\textsuperscript{24}.

**Angiogenesis, EMT, and Prostate Cancer**

The initial stages of tumor invasion require angiogenesis, the formation of new blood vessels to support tumor growth. Vascular endothelial growth factor (VEGF) signals though VEGF receptors to recruit endothelial cells for the formation of new capillaries. VEGF has been shown to be over expressed in PCa, and diets rich in tomato products have been shown to reduce VEGF levels in men with recurring PCa\textsuperscript{28,88}. Data from the Health Professionals’ Follow-Up Study demonstrated that men with higher lycopene intakes had tumors that displayed less
angiogenic potential\textsuperscript{13}. Furthermore, the inhibition of migration and invasion by lycopene was accompanied by reduced activity of matrix metalloproteinase 2 (MMP-2) and urokinase-type plasminogen activator (uPA) which are involved in extracellular matrix degradation\textsuperscript{89}. This evidence suggests that lycopene may reduce the aggressive potential of PCa by inhibiting angiogenesis. Following angiogenesis, cancer cells can migrate to new tissues and form metastases. This process is known as the epithelial to mesenchymal transition (EMT) and is a critical process for invasion and metastasis of cancer cells. EMT requires the epithelial cells to change their morphology and gene expression, assuming the shape and characteristics of mesenchymal cells. The loss of cytokeratin, E-cadherin, and cellular polarity coupled with the acquisition of a fibroblast-like shape, motility, N-cadherin, proteases, and vimentin are characteristics of cells undergoing EMT. \textit{In vivo} evidence demonstrates that tomato supplementation may reduce PCa metastases\textsuperscript{28}, but the ability of TP to reduce the initial invasive potential by inhibiting the EMT switch is under investigated.

\textbf{Caloric Restriction and Prostate Cancer}

The concept of caloric restriction, or limiting one's calories by 20-40\% of \textit{ad libitum} intake, has been suggested as a method of prolonging longevity across a multitude of species\textsuperscript{90,91}. As early as 1909, scientists noted that transplanted tumors grew slower when mice were fed a calorically restricted diet than diets \textit{ad libitum}\textsuperscript{90}. Evidence from epidemiological studies suggests that people who consume fewer calories have a lower incidence of cancer. People living in Okinawa Japan, who consume significantly fewer calories than those living on Japan’s mainland, have decreased rates of several cancers\textsuperscript{92}. Furthermore, studies on underweight populations describe significantly lower cancer rates than the general population\textsuperscript{90}. Short-term caloric restriction has also been shown to have benefits in cancer outcomes, and has been shown to
decrease the adverse effects of chemotherapeutic agents on somatic cells and enhance the cytotoxic effects of chemotherapy on cancer cells. Short term fasting in cancer patients has been shown to be safe and may help reduce side-effects from chemotherapy. Caloric-restriction, while not a new concept, warrants further investigation for cancer prevention/treatments.

**Animal Models of Caloric Restriction and Prostate Cancer**

In animal models, caloric restriction has been shown to reduce cancer incidence, improve survival, increase apoptosis, reduce proliferation, and decrease tumor burden. In Dunning R3327-H rats, 20%, 30%, and 40% caloric restriction significantly reduced tumor growth without inducing malnutrition. Furthermore, caloric restriction resulted in a less aggressive phenotype in tumor morphology, increased tumoral apoptosis, and a reduction in tumoral angiogenesis. In the TRAMP model of PCa, a 20% caloric restriction beginning at puberty at 7 weeks of age significantly reduced tumor incidence at 11 and 20 weeks of age. Interestingly, a 20% caloric restriction was still effective in reducing epithelial lesions when initiated at 20 weeks of age, though to not as great of an extent as restriction at puberty. Intermittent caloric restriction beginning at 7 weeks of age resulted in an even greater reduction in cancer incidence than chronic caloric restriction. Together, rodent studies suggest that caloric restriction suppresses PCa in rodent models.

**Pro-Inflammatory Cytokines and Prostate Cancer**

Infiltration by inflammatory cells into tumors was once thought as the immune system’s best attempt at eradicating them. Recent evidence shows that the presence of inflammatory cells contribute to tumor development and progression. Tumor–associated macrophages secrete angiogenic factors, cytokines, metalloproteinases, and other factors that contribute to tumor
growth and progression. Contributors to inflammation include interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), interferon-γ (IFN-γ), and interleukin-17 (IL-17). Elevated serum levels of IL-6 have been associated with metastatic PCa\textsuperscript{100}, but IL-6 has been suggested to play a major role in the early stages of PCa development as well\textsuperscript{101}. IL-6 may act as a growth factor and protect PCa cells from chemotherapeutic agents\textsuperscript{100} and has been shown to activate expression of AR in DU145 and LNCaP PCa cells\textsuperscript{102}. Treatment with a monoclonal antibody to IL-6 improves prognosis in patients with advanced stage PCa\textsuperscript{103}. TNF-α is a major pro-inflammatory cytokine that is secreted in the tumor micro-environment\textsuperscript{104}. An increase in prostatic TNF-α expression was associated with adverse prognostic factors after prostatectomy\textsuperscript{104}.

IL-1β is a multifunctional pro-inflammatory cytokine which has been shown to inhibit proliferation, reduce PSA, and reduce AR levels in LNCaP cells\textsuperscript{105,106}. IL-1β exerts some of its biological actions by activating the transcription factor, NF-κB\textsuperscript{105}. IL-17 produced by tumor-infiltrating lymphocytes has been implicated in tumor vascularization of cervical\textsuperscript{107} and ovarian cancers\textsuperscript{108}. IL-17 has also been suggested to promote the growth of prostate tumors from PIN\textsuperscript{109} and promote the development of CRPC in transgenic mice\textsuperscript{110}. IL-10 has been suggested to modulate tumor growth and angiogenesis\textsuperscript{111}. IL-10, an anti-inflammatory cytokine, has been shown to down-regulate levels of IL-6 and play an important role in the balance of cytokines in PCa\textsuperscript{112}. IL-10 production from cells transfected with IL-10 cDNA resulted in the down-regulation of VEGF, IL-1β, IL-6, and MMP-9 in activated macrophages which normally infiltrate tumors\textsuperscript{113}. In contrast, high circulating levels of IL-10 and IL-6 have been correlated with advanced progression and morbidity in patients with CRPC\textsuperscript{112}. The dual actions of IL-10 appear to be related to cancer stage and are important to understand. Interferons have been
proposed as the ideal tumor suppressor because they are specific in differentiating normal cells from neoplastic cells. IFN-γ activates the JAK-STAT pathway which can lead to the transcription of genes suggested to be important tumor suppressors. IFN-γ inducible genes which are located on the 10q 23-26 and 17q 21 chromosomal loci regions are deleted in 30% of PCa cases. Additionally, IFN-γ has been found to increase apoptosis and decrease tumor volumes in murine models of PCa.

Isoflavones have been shown to affect pro-inflammatory cytokines in vivo and in vitro. Genistein reduced serum expression of TNF-α and IL-6 in rats with non-alcoholic steatohepatitis, and an isoflavone-enriched soy protein diet reduced serum TNF-α and IL-1β in Zucker rats. In humans, consumption of soy nuts significantly reduced serum levels of TNF-α, but not IL-6. Genistein has been shown to reduce expression of IL-1β, TNF-α in LPS-treated microglial cells. Other studies using purified isoflavones in vitro have shown mixed results on serum levels of pro-inflammatory cytokines. There is a need to investigate soy germ and its constituents for the potential to reduce pro-inflammatory cytokines involved in prostate carcinogenesis.

**Aims of Dissertation**

There is considerable evidence describing the health benefits of carotenoids. However, when carotenoid-containing foods are consumed as part of a meal, the interactions with other nutrients in the meal can inhibit or enhance the bioavailability of carotenoids. Co-consumption with dietary fat has been shown to enhance the bioavailability of carotenoids; however, the fat type (saturation, chain length, etc.) is an important factor to consider as not all fats have the same health benefits. The specific aim for this portion of the thesis is to:
1) Determine the bioavailability of tomato carotenoids when consumed with fat sources of varying saturation and chain length.

There is a growing body of evidence that suggests tomato and soy bioactives may reduce the risk of PCa. The slow-growing nature of most PCa’s coupled with the long latency period between diagnosis and medical treatment provides an excellent window for dietary interventions to be effective in preventing the progression of PCa. The overall hypothesis of this project is that dietary bioactives from whole tomato products and from soy germ can reduce the incidence of PCa. The specific aims of this portion of the thesis are to:

2) Examine the efficacy of a standard TP to reduce the risk of PCa when administered as an intervention in a pre-clinical rodent model.

3) Determine the efficacy of whole soy germ, daidzein, and equol on the incidence of PCa in a pre-clinical rodent model.
REFERENCES


70. Hu X, Jandacek RJ, White WS. Intestinal absorption of beta-carotene ingested with a meal rich in sunflower oil or beef tallow: postprandial appearance in triacylglycerol-rich


81. Clerici C, Setchell KDR, Battezzati PM, et al. Pasta naturally enriched with isoflavone aglycons from soy germ reduces serum lipids and improves markers of cardiovascular


104. Rodríguez-Berriguete G, Sánchez-Espiridión B, Cansino JR, et al. Clinical significance of both tumor and stromal expression of components of the IL-1 and TNF-α signaling

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CHAPTER 2

Coconut Oil Enhances Tomato Carotenoid Tissue Accumulation Compared to Safflower Oil in the Mongolian Gerbil (Meriones unguiculatus)¹

ABSTRACT

Evidence suggests that monounsaturated and polyunsaturated fats facilitate greater absorption of carotenoids than saturated fats. However, the comparison of consuming a polyunsaturated fat source versus a saturated fat source on tomato carotenoid bioaccumulation has not been examined. Our goal was to determine the influence of coconut oil and safflower oil on tomato carotenoid tissue accumulation in Mongolian gerbils (Meriones unguiculatus) fed a 20% fat diet. Coconut oil feeding increased carotenoid concentrations among many compartments including total carotenoids in the serum (p=0.0003), adrenal glandular phytoene (p=0.04), hepatic phytofluene (p=0.0001), testicular all-trans lycopene (p=0.01), and cis-lycopene (p=0.006) in the prostate-seminal vesicle complex compared to safflower oil. Safflower oil-fed gerbils had greater splenic lycopene concentrations (p=0.006) compared to coconut oil-fed gerbils. Coconut oil feeding increased serum cholesterol (p=0.0001), and decreased hepatic cholesterol (p=0.0003) compared to safflower oil. In summary, coconut oil, enhanced tissue uptake of tomato carotenoids to a greater degree than safflower oil. These results may have been due to the large proportion of medium chain fatty acids in coconut oil which might have caused a shift in cholesterol flux to favor extrahepatic carotenoid tissue deposition.

INTRODUCTION

Tomato consumption has been linked to decreased chronic disease risk\(^1\). In addition to vitamins and minerals, tomatoes contain a variety of carotenoids. Lycopene is the major carotenoid found in tomatoes, but tomatoes also contain phytoene, phytofluene, \(\alpha\)-carotene, \(\zeta\)-carotene, and \(\beta\)-carotene\(^1\). Lycopene has garnered much interest because of its ability to quench singlet oxygen, attenuate LDL levels, reduce the risk of cardiovascular disease, and potentially inhibit carcinogenesis\(^2,3\). However, the other tomato carotenoids may exhibit similar bioactive properties as lycopene\(^1\). Recent studies have suggested that the combination of tomato carotenoids from whole tomato powder (TP) may be more effective in disease prevention than lycopene alone\(^1,4\).

Bioavailability describes the degree to which carotenoids may be absorbed and utilized from ingested food sources\(^2\). Many factors influence carotenoid bioavailability. The species of carotenoid, food matrix, food processing, amount of carotenoid consumed, genetic factors, and nutrient interactions as well as the co-consumption of dietary fat are all contributing factors\(^2\). The latter point was demonstrated in humans when carotenoids from a vegetable salad were more bioavailable when consumed with a full-fat salad dressing than with a reduced-fat salad dressing\(^5\). Additionally, tomato carotenoids were more readily absorbed from salsa and salad with the addition of avocado oil or avocado fruit\(^6\). Dietary fat is believed to facilitate bioavailability at the levels of solubilization, micellarization, and chylomicron packaging of dietary carotenoids\(^6–8\).

The structural properties of dietary fat also influence carotenoid bioavailability with evidence suggesting fats with differing degrees of saturation differ in their ability to enhance carotenoid absorption. In humans, saturated fat-rich beef tallow increased post-prandial \(\beta\)-
carotene content in chylomicrons compared to polyunsaturated fat-rich sunflower oil\textsuperscript{9}. However, butter, a source of saturated fat, reduced the post-prandial chylomicron response in humans when compared with monounsaturated olive oil and polyunsaturated sunflower oil\textsuperscript{10}. These studies suggest that monounsaturated and polyunsaturated fats enhance secretion of chylomicrons, while saturated fat enhances carotene incorporation into chylomicrons. The specific effects, however, of a saturated vs. polyunsaturated fat on tomato carotenoid incorporation into chylomicrons has not been previously reported.

Previous studies have indicated that fatty acid chain length may also influence carotenoid bioavailability and absorption. Borel et al. demonstrated increased solubility of carotenoids as fatty acid chain length decreased\textsuperscript{8}. Lycopene and β-carotene were more soluble in tricaprylin, a source of medium chain triglycerides, when compared to long chain triglycerides from fish oil\textsuperscript{8}. Incorporation of carotenoids into mixed micelles was enhanced by the addition of long chain fatty acids \textit{in vitro} when compared to medium chain fatty acids\textsuperscript{11}. However, carotenoid uptake into Caco-2 cells was not impacted by fatty acyl composition of mixed micelles\textsuperscript{11}, suggesting triglyceride chain length does not affect carotenoid uptake by enterocytes. Furthermore, absorption of β-carotene was similar when medium chain triglycerides and long chain triglycerides were compared in the perfused, isolated rat intestine\textsuperscript{12}. Borel et al. also observed an increase in β-carotene in chylomicrons when humans ingested a salad with long chain triglycerides compared to medium chain triglycerides\textsuperscript{13}. In short, while medium chain triglycerides are better at solubilizing lycopene and β-carotene than long chain triglycerides, human and animal trials suggest long chain fatty acids may facilitate micellarization and incorporation of carotenes into chylomicrons. Intestinal absorption of carotenes does not seem to be significantly impacted by fatty acid chain length. While these reports describe the impact of
fatty acid chain length and saturation on specific underlying steps of carotenoid bioavailability, there is little reported on the summative outcome of these altered mechanisms in terms of resultant serum and tissue carotenoid concentrations in response to differing consumed fat types.

Epidemiological evidence suggests regular intake of carotenoid-containing foods, such as fruits and vegetables, may decrease the risk for certain cancers\(^3\). A consequence of increased bioavailability may be increased bioaccumulation. Tissues with increased carotenoid bioaccumulation may offer some enhanced protection against oxidation and/or other events that lead to cancer.

The Mongolian gerbil (Meriones unguiculatus) has been shown to absorb β-carotene and lycopene intact\(^{14}\). In addition we have reported that other tomato carotenoids, phytoene and phytofluene, have marked accumulation in liver, spleen, lungs, testes, adrenal glands, the prostate-seminal vesicle complex, and adipose tissues\(^{15}\). The Mongolian gerbil is an excellent model for studies involving carotenoid bioaccumulation because it accumulates tomato carotenoids in levels proportionate to humans\(^{15}\).

Previously, we have fed 10% TP diets to mice, rats, and gerbils\(^4,15–17\). Gerbils fed a 10% TP diet for 26 days followed by a 2 day wash-out period, had serum lycopene levels of 0.007 μmol/dL\(^{15}\). Higher serum lycopene concentrations have been observed in men ages 19-50 years old with an average serum lycopene concentration of 0.05 μmol/dL\(^{18}\). Therefore, a 10% TP rodent diet can be utilized based upon having similar physiologically relevant serum lycopene concentrations to those seen in humans.

In addition to carotenoid accumulation, the lipoprotein profiles of the gerbil are more similar to humans than most other rodents, making carotenoid metabolism findings more relevant than those results in other species\(^{19}\). A variety of fats have been successfully fed to
gerbils to study lipid metabolism. In particular, two very different fats, saturated coconut oil and polyunsaturated safflower oil were previously fed to gerbils to study lipoprotein metabolism\textsuperscript{20,21}. Coconut oil is a good source of medium chain triglycerides commonly used in enteral feeding solutions for humans\textsuperscript{22}. Safflower oil is a common polyunsaturated fat used for cooking and as an ingredient in salad dressings\textsuperscript{23}.

How fatty acid chain length impacts the absorption and bioaccumulation of the array of tomato carotenoids, including phytoene, phytofluene, ζ-carotene, β-carotene, and lycopene, from tomatoes has not been determined. We chose coconut oil and safflower oil to study carotenoid lipid metabolism because of the use of these fats in previous studies\textsuperscript{20,21} and the differing fat characteristics which may impact carotenoid accumulation. Therefore this work compares the effect of medium chain triglyceride-rich coconut oil vs. long chain triglyceride-rich safflower oil on tissue bioaccumulation of tomato carotenoids from whole TP in Mongolian gerbils.

**MATERIALS AND METHODS**

**Chemicals.** The HPLC-grade solvents, hexane, methanol, dichloromethane, methyl-tert butyl ether (MTBE), and chloroform were purchased from Fisher Scientific (Pittsburg, PA). Potassium hydroxide and ammonium acetate were purchased from Fisher Scientific. Ethanol and polyoxyethylene octylphenyl ether were purchased from Sigma-Aldrich (St. Louis, MO). Phytoene, phytofluene, and ζ-carotene standards were purchased from Carotenature (Lupsingen, Switzerland). Lycopene and β-carotene were extracted from lycopene or β-carotene beadlets and obtained from DSM (Heerlen, Netherlands).

**Animal Methods.** The University of Illinois Institutional Animal Care and Use Committee (IACUC) approved all procedures with animals. Forty-day-old male Mongolian gerbils were obtained from Charles River Laboratory (Wilmington, MA) \(n=40\) and were fed a pelleted chow
diet for a 2-day acclimation period. On day 3, gerbils were randomized to either one of two control diets (n=10/dietary treatment). Diets were modified from previously used high fat gerbil diets\textsuperscript{19} and those used to deliver TP in the diet to rodents\textsuperscript{15} (Table 2.1). Control diets consisted of 20\% safflower oil (SOC) or 18\% coconut oil, with 2\% safflower oil (COC) to prevent essential fatty acid deficiency\textsuperscript{24}. Experimental diets consisted of 20\% safflower oil with 10\% TP (TPSO) or 18\% coconut oil and 2\% safflower oil with 10\% TP (TPCO). TP contributed lycopene, β-carotene, phytoene, phytofluene, and ζ-carotene to the diet (Table 2.2). The fatty acid profile for each diet is outlined in Table 3. Coconut oil consisted of 42.5\% lauric (C12:0), 15.8\% myristic (C14:0), and 8.9\% palmitic acids (C16:0). Minor components in coconut oil included oleic acid (C18:1) (7.8\%), and linoleic acid (C18:2) (9.3\%). The fatty acid profile of safflower oil consisted of 67.8\% linoleic acid, and 17.2\% oleic acid. Minor contributors included 6.4\% palmitic acid (C16:0) and 2.6\% stearic acid (C18:0) (Table 2.3). All gerbils were fed 15 g of the respective diet every two days for 28 days. Gerbil weights were recorded every two days throughout the study to monitor health and growth. On day 30, all four groups were fasted for approximately 4-6 hours before being sacrificed. Gerbils were anesthetized with CO\textsubscript{2}, blood was collected via cardiac puncture and gerbils were then euthanized via CO\textsubscript{2} asphyxiation. Following euthanization, animals were partially shaved and ventral skin samples were taken from each animal. Skin, gonadal adipose, heart, lungs, testes, adrenal glands, and prostate seminal vesicle complex were removed, weighed, flash frozen in liquid nitrogen and stored at -80 °C for future analysis. Additionally, livers were perfused with ice cold 1.15\% potassium chloride (KCl) before being frozen and stored.

**Dietary Carotenoid Extraction.** To verify dietary carotenoid content, the entire extraction procedure was performed underneath yellow light along with other general
precautions taken to preserve carotenoids. The extraction method used has been previously described\textsuperscript{16}. First, 0.025 g of powdered diet was suspended in 5 mL of ethanol containing 0.1% butylated hydroxytoluene (BHT). Samples were homogenized at level 3 (Power Gen 500, Fisher-Scientific, Pittsburg, PA) for 120 s and 1 mL of saturated potassium hydroxide (KOH) solution was added. Samples were saponified in a 60 °C water bath with intermittent vortexing for 30 min, were removed, and 2 mL of deionized water and 6 mL of hexanes were added for biphasic extraction of carotenoids. Samples were vortexed, separated by centrifugation for 10 minutes and the organic hexanes phase was removed and reserved. The addition of hexanes, followed by separation and removal was repeated two more times. Hexanes were pooled and concentrated using a Speedvac concentrator (Speedvac model AS160, Savant, Milford, MA). Samples were stored under argon at -20 °C for less than 48 hours before HPLC analysis.

**Tissue Carotenoid Extraction.** Liver carotenoid concentrations were determined by extracting approximately 0.1 g of liver tissue. Whole spleen (~ 0.07 g) and prostate-seminal vesicle complex (~ 0.4 g) were extracted for each animal. For adrenal glands (~ 0.03 g), testes (~ 1.1 g), and lungs (~ 0.4 g), both specimens were pooled and extracted together for each animal. Samples were added to 5 mL of ethanol containing 0.1% BHT followed by manual homogenization. Carotenoids were extracted by hexanes using the above-described protocol.

**Skin Carotenoid Extraction.** Subcutaneous fat was removed prior to the extraction. The skin extraction procedure was previously described\textsuperscript{25}. Briefly, ~ 0.3 g of skin was flash frozen and ground into a fine powder, then 1 mL of ethanol with 0.1% BHT, 1 mL of water, and 5 mL of hexane/dichloromethane (5:1, v/v) were added to the samples. The samples were mixed and the top layer of hexane and dichloromethane was removed. The extraction was repeated and both
hexane and dichloromethane extractions were pooled and dried under argon gas. Samples were stored at -20 °C for less than 24 hours before analysis.

**HPLC Analysis.** Samples were analyzed using high-pressure liquid chromatography – photodiode array detector system (HPLC-PDA, Waters, Milford, Ma). A C30 reversed phase column (4.6 x 150 mm, 3 μm, YMC, Wilmington, NC) cooled to 18 °C was used with MTBE, methanol, and 1.5% (w/v) aqueous ammonium acetate mobile phases to separate phytoene, phytofluene, lycopene, ζ-carotene, and β-carotene. Specific mobile phase compositions and gradient elution method were used as described previously. Extracts of all tissues, except for the liver were reconstituted in 40 μL of MTBE. Liver extracts were reconstituted in 300 μL of MTBE. Twenty-seven μL of the sample reconstituted in MTBE was injected for each tissue or diet for carotenoid analysis. Carotenoids were identified and quantified via UV spectra, retention times and standard comparison. Phytoene, phytofluene, and ζ-carotene standards were purchased and lycopene was extracted from 10% Redivivo beadlets. β-carotene was extracted from 15% beadlets. Samples were analyzed within 48 hours of extraction. Samples were kept at 4 °C on an autosampler cooling tray and handled under yellow light to reduce carotenoid degradation.

**Hepatic Lipid Extraction.** Total liver lipids were extracted following the Folch method. Briefly, 0.5 g of tissue was homogenized in a solution of chloroform: methanol (1:1). A 0.29% sodium chloride (NaCl) solution was added to promote phase separation, followed by centrifugation. Chloroform was added followed by centrifugation and addition of NaCl solution. This step was repeated twice. Supernatant was retained after each of the steps, pooled into pre-weighed tubes, and evaporated in a fume hood for approximately 48 hours, before being stored in a desiccator for 24 hours. Samples were then weighed to determine total lipid concentration.
Serum and Hepatic Cholesterol Analysis. Serum total cholesterol concentrations were quantified using Wako Cholesterol E enzymatic colorimetric assay (Wako Chemicals, Richmond, VA). The protocol was modified to the manufacturer’s microtiter procedure, using 3 µL of serum per analysis (Wako Chemicals, Richmond, VA). Hepatic cholesterol concentrations were determined by reconstitution of extracted hepatic lipids in isopropyl alcohol containing 10% polyoxyethylene octylphenyl ether. All standards were diluted with the same extraction solution and the remainder of the analysis was performed following the manufacturer’s protocol.

Fatty Acid Analysis. Fatty acid analysis was previously described. Briefly, 0.1 g of diet was weighed and 2 mL of a dilute internal standard, tridecanoic acid, was added. Methanolic-HCl was added and the samples were incubated at 100 °C for an hour. Two mL of hexanes and 5 mL of potassium carbonate were slowly added to each tube and the samples were vortexed and centrifuged. The top organic layer was collected, dried under nitrogen gas, and protected from light. The samples were then transferred to gas chromatography vials and analyzed using gas chromatography as previously described.

Statistical Analyses. Tissue carotenoid concentrations in the safflower oil + TP and coconut oil + TP groups were compared using ANOVA, and significant differences were detected using Tukey’s Studentized test (α=0.05) when the assumptions of ANOVA were met. When the assumptions of ANOVA were not met, the Wilcoxon test was used to detect differences (α=0.05). Statistical analysis was performed using SAS v. 7.1 (SAS Institute Inc., Cary, NC). Data are reported as mean ± standard error of the mean (SEM).

RESULTS

Body Weight and Food Intake. The final weight of gerbils at sacrifice did not differ by dietary treatment group (p=0.41) with an average gerbil weight of 69.6 ± 0.8 g. Additionally, the
final weight of gerbil livers did not differ by dietary treatment (p=0.15). When compared as percent of body weight, there were some minor organ weight differences between groups. The final weight of gonadal-adipose tissue were different between the safflower oil + TP-fed and coconut oil-fed dietary groups (p=0.03), with an average weight of 0.90 ± 0.05 g and 0.68 ± 0.05 g, respectively. The final weight of the testes was greater (p=0.02) in the safflower oil + TP-fed group 1.15 ± 0.03 g, compared to the other dietary groups 1.07 ± 0.02 g. Safflower oil-fed animals had greater (p=0.04) adrenal glandular weights (0.05 ± 0.001 g) compared to coconut oil-fed animals (0.038 ± 0.001 g). In short, gonadal adipose, testes, and adrenal glandular weights were significantly greater in safflower oil-fed animals which might suggest greater accumulation of fat in these tissues. These fat-specific findings have not been previously reported.

Diet Carotenoid Profile. Lycopene was also the most prevalent carotenoid in the TP; accounting for over 90% of the total carotenoid content (Table 2.2). The TP diet also contained phytoene, phytofluene, ζ-carotene, and β-carotene.

Tissue Carotenoid Accumulation. Lycopene was the dominant carotenoid in the liver, spleen, testes, lung, serum, and skin. Phytoene was the most abundant carotenoid in the adrenal glands. There was no accumulation of phytoene in the androgen-sensitive tissues, the testes or prostate-seminal vesicle complex. Coconut oil feeding increased pulmonary phytofluene (p=0.006) and ζ-carotene (p=0.003), adrenal glandular phytoene (p=0.05), hepatic phytoene (p=0.02) and phytofluene (p=0.0001), and testicular phytofluene (p=0.01), cis-lycopene (p=0.01), all-trans lycopene (p=0.010), total lycopene (p=0.01) and ζ-carotene (p=0.02). Coconut oil feeding also increased phytofluene (p=0.008), cis-lycopene (p=0.006), and ζ-carotene (p=0.005) in the prostate-seminal vesicle complex. The prostate-seminal vesicle complex (p=0.01), testes
(p=0.01), and serum (p=0.0003) had greater total carotenoid concentrations in animals fed coconut oil (Table 2.4). Safflower oil-fed gerbils had greater splenic all-trans lycopene (p=0.03), and total lycopene (p=0.006) compared to coconut oil-fed animals.

To our knowledge, this is the first time tomato carotenoids have been reported in skin from Mongolian gerbils (Table 2.4). All-trans lycopene and cis-lycopene were consistently detected in skin in quantifiable amounts, but differences between groups were not significant (p=0.17) and (p=0.38) respectively. Phytoene and phytol were detected in skin in fewer than 50% of the animals. Phytoene and phytol were considered detectable, but unquantifiable if HPLC signals corresponded with carotenoid concentrations in the injection carrier solvent of less than 0.2 nM. In the skin, ζ-carotene and β-carotene were detected in fewer than 20% of the animals. When detected, ζ-carotene and β-carotene were in amounts below the level of quantification (0.9 nM ζ-carotene, 0.1 nM β-carotene in the HPLC carrier solvent). β-carotene was detected but unquantifiable in most other tissue extracts. An unidentified compound was found in the skin of animals fed TP diets and in those fed control diets. The compound had a similar retention time and maximum absorbance (282.8 nm) to the phytoene standard (286 nm). However, upon closer inspection, the compound did not have the characteristic spectral fine structure of phytoene determined by comparison to phytoene standards. Therefore this compound was determined not to be phytoene.

**Hepatic Lipids.** Total hepatic lipid concentrations did not differ by dietary treatment group (p=0.9) with an average concentration of 37 ± 0.8 mg/g for all groups.

**Serum and Hepatic Cholesterol.** Animals fed coconut oil diets maintained significantly higher serum cholesterol concentrations (178 ± 8.2 mg/dL) than animals fed safflower oil diets (82 ± 6.7 mg/dL) (p=0.0001) (Figure 2.1). In the current study, animals fed safflower oil had
significantly higher (p=0.0003) hepatic cholesterol concentrations (4.0 ± 0.2 mg/g) compared to coconut oil fed groups (2.7 ± 0.14 mg/g) (Figure 2.2). TP addition to either safflower or coconut oil diets did not alter hepatic cholesterol concentrations (p=0.4 and p=0.37, respectively).

**DISCUSSION**

The aim of this study was to compare the effects of an oil high in medium chain saturated fatty acids (coconut oil) with an oil high in long chain polyunsaturated fat (safflower oil) on tomato carotenoid tissue accumulation in gerbils. We observed enhanced tissue bioaccumulation of tomato carotenoids in all tissues from animals fed coconut oil except for the spleen and skin as well as differential tissue accumulation between carotenoids unrelated to dietary fat among the tissues measured.

Carotenoids are bioactive molecules with antioxidant capabilities and may function to reduce the risk for certain chronic diseases and cancers\(^3\). Lycopene, phytoene, and phytofluene have been shown to accumulate in a variety of human and animal tissues including liver, adrenal glands, testes, kidney, pancreas, breast, skin, ovary, spleen, and prostate\(^1,3\). In the current study, tissue concentrations of phytoene, phytofluene, *cis*-lycopene, *all-trans* lycopene, *ζ*-carotene, and *β*-carotene were quantified in liver, lungs, spleen, adrenal glands, testes, prostate-seminal vesicle complex, serum, and skin (Table 2.4).

Previously, we have shown that tomato carotenoids differentially accumulate in both male Fisher 344 rat and Mongolian gerbil tissues in levels disproportionate to those fed in the diet\(^15,16\). In the current study, lycopene was the dominant carotenoid in the liver, spleen, testes, lung, serum, and skin. Lycopene was also the most prevalent carotenoid in the TP, accounting for over 90% of the total carotenoid content (Table 2.2). Phytoene was the most abundant carotenoid in the adrenal glands accounting for 62% of total carotenoids in the coconut oil-fed and 45% in the
safflower oil-fed adrenal glands, despite the fact that phytoene concentrations in the diet contributed only 2% of the total carotenoids. These results suggest tissue-specific uptake or metabolism of tomato carotenoids consistent with reports from our previous studies\textsuperscript{15,16}. Similar to findings by Engelmann, there was no accumulation of phytoene in the androgen-sensitive tissues, which suggests a difference in absorption or metabolism of phytoene in these tissues\textsuperscript{15}.

Tissue accumulation of carotenoids was affected by type of fat consumed with coconut oil increasing carotenoid accumulation in almost every tissue except for the spleen and skin. Splenic lycopene was significantly increased by the safflower oil diet. Previous studies have observed carotenoid accumulation levels in the spleen to be relatively high compared to other tissues\textsuperscript{15}. The spleen is important for immune function because it removes bacteria and debris from the bloodstream\textsuperscript{29}. However, carotenoid enrichment of the spleen is poorly understood.

We observed differences in carotenoid accumulation by dietary fat type in the prostate-seminal vesicle complex. Phytofluene was the dominant carotenoid in the prostate-seminal vesicle complex of the coconut oil-fed animals accounting for 50% of the total carotenoids. In contrast, lycopene was the dominant carotenoid in the prostate-seminal vesicle complex of the safflower oil-fed animals accounting for 57% of the total carotenoids. Engelmann observed lycopene as the dominant carotenoid in the prostate-seminal vesicle complex when gerbils were fed cottonseed oil, which contains a mixture of saturated, monounsaturated, and polyunsaturated fat\textsuperscript{15}. This suggests lycopene accumulation in the prostate-seminal vesicle complex is dependent on the type of fat consumed. Polyunsaturated fats may enhance lycopene accumulation in the prostate-seminal vesicle complex, while saturated fat from coconut oil, enhances total carotenoid accumulation in the prostate-seminal vesicle complex with phytofluene being the major carotenoid deposited.
The testes of the coconut oil-fed animals had greater total lycopene concentrations, but weighed significantly less than those of the safflower oil-fed animals. Studies using carotenoid metabolizing enzyme knockout mice have shown that dietary lycopene and TP reduce testicular testosterone which might explain alterations in growth of the testes.\textsuperscript{17}

To evaluate if the change in carotenoid tissue deposition was a result of a change in cholesterol and lipid metabolism, serum and hepatic cholesterol and hepatic lipids were quantified. Animals fed coconut oil diets had significantly higher serum cholesterol concentrations than animals fed safflower oil diets (Figure 2.1). Coconut oil is known to increase serum cholesterol in humans due to its high amounts of lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids.\textsuperscript{30} The primary fatty acid in coconut oil, lauric acid, accounted for 42\% of the fatty acids in the diet of the coconut oil-fed animals (Table 2.3). The combination of these three cholesterol-raising fatty acids in coconut oil likely contributed to the increased serum cholesterol in the current study.

In the current study, animals fed safflower oil had significantly higher (p=0.0003) hepatic cholesterol concentrations compared to coconut oil fed groups (Figure 2.2). This finding is consistent with other studies showing that polyunsaturated fats increase hepatic cholesterol uptake.\textsuperscript{31,32} In contrast to coconut oil, safflower oil is composed of long chain polyunsaturated fatty acids, primarily linoleic acid (C18:2) (Table 2.3).

Saturated fats have long been grouped together for dietary recommendations, but they may not all have the same biological impact. Coconut oil is a unique saturated fat because of its fatty acid profile. The fatty acid profiles of saturated fats can be dramatically different; for instance butter is primarily composed of short chain fatty acids, coconut oil is primarily medium chain fatty acids, and beef tallow consists of long chain fatty acids.\textsuperscript{23} Studies comparing coconut
oil to other saturated fats have conflicting results concerning serum cholesterol. When coconut oil and beef tallow were compared in humans, coconut oil increased serum LDL and total cholesterol relative to beef tallow\textsuperscript{33}. Similarly, long chain saturated fatty acids fed to guinea pigs resulted in decreased plasma LDL concentrations when compared to coconut oil\textsuperscript{34}. However, butter significantly increased LDL and total cholesterol levels when compared to coconut oil in moderately hypercholesterolemic adults\textsuperscript{35}. Differences in saturated fatty acid chain length might account for differences in LDL metabolism and LDL receptor synthesis; therefore we cannot categorize the effect of all saturated fats on plasma cholesterol based on fatty acid saturation alone.

The composition of fatty acids in oils can alter carotenoid solubulization, micellarization, absorption, and chylomicron formation. While in the current study, carotenoid absorption was not measured directly, fatty acid chain length does not appear to contribute to differences in carotenoid absorption when comparing different fats\textsuperscript{12}. In the current study, carotenoid tissue accumulation was increased in animals fed coconut oil. Previously, Borel et al. observed increased solubility of carotenoids with decreasing fatty acid chain length\textsuperscript{8}. Carotenoids are lipophilic compounds and once released from the food matrix, are solubilized into mixed micelles with bile salts. Medium chain fatty acids in coconut oil may cause increased carotenoid solubility into mixed micelles compared to the long chain fatty acids from safflower oil.

Previous studies have indicated increased $\beta$-carotene and lycopene micellarization with the addition of long chain triglycerides\textsuperscript{11}. However, the current study found coconut oil, a source of medium chain fatty acids, increased carotenoid tissue deposition when compared to a long chain polyunsaturated fat, safflower oil. In contrast to the current study, this previous study used tricaprylin as a source of medium chain fatty acids, which is high in caprylic acid (C8:0)\textsuperscript{11}. 
Caprylic acid is shorter than the primary fatty acids found in coconut oil. Relative to tricaprylin, longer chain medium chain fatty acids in coconut oil may enhance the carotenoid absorption properties to a greater degree than shorter chain medium chain fatty acids and to the same extent that long chain fatty acids do. The comparison of different medium chain fatty acids’ effects on carotenoid metabolism has not been determined, however, the composition of medium chain triglycerides are important because of the potential impact chain length has on micellarization and incorporation of carotenoids into chylomicrons.

Previous studies have shown increased incorporation of β-carotene and lycopene into chylomicrons when long chain triglycerides were used\textsuperscript{13}. However, in the current study, coconut oil facilitated carotenoid tissue accumulation to a greater degree than safflower oil. Because coconut oil is composed of medium chain fatty acids, these fatty acids can bypass chylomicron formation and be absorbed directly by the portal vein\textsuperscript{13}. This results in decreased post-prandial chylomicron formation with medium chain fatty acids\textsuperscript{13}. Additionally, it has been described that medium chain triglycerides can be incorporated into chylomicrons when they are the only source of fat in the diet\textsuperscript{36}. In the current study, gerbils were fed a 20% fat diet of 18% coconut oil and 2% safflower oil or 20% safflower oil. The high amount of medium chain triglycerides in coconut oil may have facilitated incorporation into mixed micelles and absorption by enterocytes, however the mechanism of carotenoid absorption is unclear.

We observed increased tissue accumulation of tomato carotenoids in every tissue measured except for the spleen and skin in animals fed coconut oil. Chylomicrons are eventually transported to the liver as chylomicron remnants where hepatic metabolism of carotenoids can occur\textsuperscript{17}. In the liver, carotenoids are repackaged into very low-density lipoproteins (VLDL) and released into circulation where they have access to peripheral tissues. While in circulation,
VLDLs lose triglycerides and become denser lipoproteins: intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL). LDLs and IDLs are returned to the liver where 80% of the LDL is catabolized\textsuperscript{37}. Saturated fats can interrupt this cycle by reducing hepatic uptake of LDL\textsuperscript{38,39}. The primary fatty acids in coconut oil (lauric, myristic and palmitic acids) reduce LDL receptor activity\textsuperscript{30,39}, thus increasing serum cholesterol and leaving more LDL free to recirculate to peripheral tissues. Non-polar carotenes, specifically lycopene, are carried primarily in the LDL fraction of lipoproteins\textsuperscript{40}. In the current study, serum cholesterol in the coconut oil-fed animals was significantly increased and hepatic cholesterol was significantly decreased when compared to safflower oil-fed animals. Perhaps a coconut oil-mediated change in LDL levels altered the flux of carotenoids between the liver and peripheral tissues resulting in increased tissue-specific accumulation of carotenoids and/or decreased clearance of carotenoids by the liver.

However, when considering the differential accumulation of tissue carotenoids by dietary fat (Table 2.4), in tissues enriched with carotenoids by coconut oil feeding, there might be a concurrent shift in tissue fatty acid profile to one more similar to that of coconut oil. Perhaps tissues that accumulated tomato carotenoids preferentially utilize medium chain fatty acids, and as a result of increased tomato carotenoid solubility in medium chain fatty acids\textsuperscript{8}, carotenoid accumulation was greater. While tissue fatty acid profiles were not measured in the current study, studies in dairy cattle have shown that dietary fatty acid manipulation can significantly shift the fatty acid profiles of muscle and milk\textsuperscript{41}. Further insight into the interaction between dietary fat and tissue carotenoid accumulation may be gained by examining tissue fatty acid profiles.
**Carotenoid stability in the diet.** Polyunsaturated fats are unstable because their double bonds are susceptible to oxidation\(^ {38} \). To account for possible fat-specific carotenoid degradation, carotenoid stability in the diet was measured. Carotenoid content of the diet was measured prior to being exposed for 2-days in the animal facility and again after exposure. There was no difference in carotenoid content of the diet due to oil type during the 2-day exposure period (data not shown). Thus the differences in carotenoid accumulation are not the result of carotenoid degradation in the diet.

In summary, differential accumulation of tomato carotenoids in liver, spleen, lungs, adrenal glands, testes, prostate-seminal vesicle complex, serum, and skin was observed independent of fat type in the Mongolian gerbil. In addition, we observed fat type-specific effects on carotenoid accumulation. Coconut oil feeding resulted in increased tomato carotenoid bioaccumulation compared to safflower oil in all tissues measured but the spleen and skin. Increased tissue accumulation may have been a result of increased solubility of tomato carotenoids in the intestinal lumen, portal absorption of medium chain fatty acids, a cholesterol-mediated change in the flux of carotenoids between the liver and peripheral tissues, facilitated carotenoid cellular uptake by specific fatty acids, or the combination of the four. Research investigating the influence of different saturated fats of differing chain lengths on tomato carotenoid accumulation and cholesterol flux should be undertaken. It is important to understand the differential accumulation patterns of carotenoids in specific tissues and how dietary fats can affect these profiles when carrying out animal or human feeding trials with carotenoids.
ABBREVIATIONS USED

COC, coconut oil; TPCO, coconut oil + 10% TP; SOC, safflower oil; TPSO, safflower oil + 10% TP; BHT, butylated hydroxytoluene; HPLC-PDA, high performance liquid chromatography-photodiode array.

ACKNOWLEDGMENTS

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### FIGURES AND TABLES

**Table 2.1. Gerbil Diet Composition Table**

<table>
<thead>
<tr>
<th>Component</th>
<th>18% Coconut Oil and 2% Safflower Oil Control Diet</th>
<th>20% Safflower Oil Control Diet</th>
<th>18% Coconut Oil and 2% Safflower Oil + 10% TP Diet</th>
<th>20% Safflower Oil +10% TP Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>283</td>
<td>283</td>
<td>242</td>
<td>242</td>
</tr>
<tr>
<td>Casein (\text{a})</td>
<td>200</td>
<td>200</td>
<td>167</td>
<td>167</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>119</td>
<td>119</td>
<td>119</td>
<td>119</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>94</td>
<td>94</td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>Cellulose</td>
<td>60</td>
<td>60</td>
<td>43.8</td>
<td>43.8</td>
</tr>
<tr>
<td>Micronutrients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineral Mix (\text{b})</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin Mix (\text{c})</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>L-cystine</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>CaHPO(_4)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>MgO</td>
<td>1.75</td>
<td>1.75</td>
<td>1.75</td>
<td>1.75</td>
</tr>
<tr>
<td>TP (\text{d})</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Coconut Oil</td>
<td>180</td>
<td>0</td>
<td>180</td>
<td>0</td>
</tr>
<tr>
<td>Safflower Oil</td>
<td>20</td>
<td>200</td>
<td>20</td>
<td>200</td>
</tr>
<tr>
<td>kcal/g</td>
<td>3.73</td>
<td>3.73</td>
<td>3.73</td>
<td>3.73</td>
</tr>
</tbody>
</table>

\(\text{a}\) Vitamin-free test casein contains 0.9 g protein/g casein.

\(\text{b}\) AIN-93G-MX (Teklad) contains calcium carbonate (12.495 g/kg diet); potassium phosphate, monobasic (6.86 g/kg diet); potassium citrate, monohydrate (2.48 g/kg diet); sodium chloride (2.59 g/kg diet); potassium sulfate (1.63 g/kg diet); magnesium oxide (0.85 g/kg diet); ferric citrate (0.21 g/kg diet); zinc carbonate (0.06 g/kg diet); manganese carbonate (0.02 g/kg diet); cupric carbonate (0.01 g/kg diet); potassium iodate (0.35 mg/kg diet); sodium selenate (0.36 mg/kg diet); ammonium paramolybdate, tetrahydrate (0.28 mg/kg diet); sodium meta-silicate, nonahydrate (0.0575 g/kg diet); chromium potassium sulfate, doceahydrate (9.6 mg/kg diet); lithium chloride (0.609 mg/kg diet); boric acid (2.85 mg/kg diet); sodium fluoride (2.22 mg/kg diet).
diet); nickel carbonate hydroxide, tetrahydrate (0.111 mg/kg diet); ammonium meta-vanadate (0.231 mg/kg diet); and sucrose (7.73 g/kg diet)\textsuperscript{36}.

\textsuperscript{c} AIN-93-VX (Teklad) provides niacin (0.015 g/kg diet); calcium pantothenate (0.008 g/kg diet); pyridoxine HCl (3.5 E-3 g/kg diet); thiamin HCl (3 E-3 g/kg diet); riboflavin (3 E-3 g/kg diet); folic acid (1 E-3 g/kg diet); biotin (0.1 mg/kg diet); vitamin B12 (0.1\% in mannitol) (0.0125 g/kg diet); vitamin E, DL-alpha tocopheryl acetate (500 IU/g) (0.075 g/kg diet); vitamin A palmitate (500,000 IU/g) (0.004 g/kg diet); vitamin D3 cholecalciferol (500,000 IU/g) (0.001 g/kg diet); vitamin K1, phylloquinone (0.375 mg/kg diet); sucrose (4.87 g/kg diet)\textsuperscript{36}.

\textsuperscript{d} Drum dried TP (Futureceuticals, Momence, IL) contains 3.68 kcal/g, 0.01 g protein/g, 0.03 g fat/g, 0.16 g fiber/g, and 0.52 g carbohydrate/g.
Table 2.2. Diet Carotenoid Concentrations (µmol/Kg diet)\(^a\)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Safflower Oil + 10% TP Mean ± SEM</th>
<th>18% Coconut Oil and 2% Safflower Oil + 10% TP Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoene</td>
<td>12.2 ± 1.6</td>
<td>11.5 ± 1.4</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>5.1 ± 0.6</td>
<td>6 ± 0.3</td>
</tr>
<tr>
<td>\textit{cis}-Lycopene</td>
<td>86.2 ± 8.8</td>
<td>81 ± 9.1</td>
</tr>
<tr>
<td>\textit{all-trans} Lycopene</td>
<td>282 ± 47</td>
<td>272 ± 40</td>
</tr>
<tr>
<td>Total Lycopene</td>
<td>368 ± 55</td>
<td>353 ± 49</td>
</tr>
<tr>
<td>(\zeta)-Carotene</td>
<td>1.5 ± 0.09</td>
<td>1.6 ± 0.07</td>
</tr>
<tr>
<td>(\beta)-Carotene</td>
<td>1.2 ± 0.5</td>
<td>1.5 ± 0.6</td>
</tr>
</tbody>
</table>

\(^a\) Diet carotenoid concentrations for 10% TP + 20% safflower oil diet and 10% TP + 18% coconut oil and 2% safflower oil. Values represent the mean of diet samples analyzed in triplicate ± SEM.
### Table 2.3. Fatty Acid Analysis of dietary fats

<table>
<thead>
<tr>
<th>Fatty Acid Chain Length</th>
<th>Common name</th>
<th>COC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>TPCO&lt;sup&gt;c&lt;/sup&gt;</th>
<th>SOC&lt;sup&gt;d&lt;/sup&gt;</th>
<th>TPSO&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:0</td>
<td>caprylic</td>
<td>4.7%</td>
<td>3.9%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>10:0</td>
<td>capric</td>
<td>5.2%</td>
<td>4.9%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>12:0</td>
<td>lauric</td>
<td>42.9%</td>
<td>42.2%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>14:0</td>
<td>myristic</td>
<td>15.8%</td>
<td>15.9%</td>
<td>0.2%</td>
<td>0.2%</td>
</tr>
<tr>
<td>16:0</td>
<td>palmitic</td>
<td>8.9%</td>
<td>8.9%</td>
<td>6.3%</td>
<td>6.5%</td>
</tr>
<tr>
<td>18:0</td>
<td>stearic</td>
<td>3.0%</td>
<td>3.1%</td>
<td>2.6%</td>
<td>2.6%</td>
</tr>
<tr>
<td>18:1</td>
<td>oleic</td>
<td>7.7%</td>
<td>7.9%</td>
<td>17.2%</td>
<td>17.1%</td>
</tr>
<tr>
<td>18:2</td>
<td>linoleic</td>
<td>9.0%</td>
<td>9.6%</td>
<td>68.0%</td>
<td>67.5%</td>
</tr>
<tr>
<td>18:3</td>
<td>linolenic</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.2%</td>
<td>0.2%</td>
</tr>
<tr>
<td>20:0</td>
<td>arachidic</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.4%</td>
<td>0.3%</td>
</tr>
<tr>
<td>20:1</td>
<td>gondoic</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.2%</td>
<td>0.2%</td>
</tr>
<tr>
<td>22:0</td>
<td>behenic</td>
<td>0.1%</td>
<td>0.3%</td>
<td>0.0%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Total unidentified FA</td>
<td></td>
<td>2.2%</td>
<td>2.9%</td>
<td>2.7%</td>
<td>3.2%</td>
</tr>
<tr>
<td>Total MCFA</td>
<td></td>
<td>52.8%</td>
<td>51.0%</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Total LCFA</td>
<td></td>
<td>44.6%</td>
<td>46.0%</td>
<td>95.2%</td>
<td>94.9%</td>
</tr>
<tr>
<td>Total Fatty acids</td>
<td></td>
<td>99.7%</td>
<td>99.9%</td>
<td>98.0%</td>
<td>98.3%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Table 3 represents the percentage of fatty acids based on total fatty acids in the diet run in duplicates.

<sup>b</sup> COC represents 18% coconut oil and 2% safflower oil

<sup>c</sup> TPCO represents 18% coconut oil and 2% safflower oil + 10% TP

<sup>d</sup> SOC represents safflower oil

<sup>e</sup> TPSO represents safflower oil + 10% TP
Table 2.4. The Impact of Dietary Fat on Carotenoid Accumulation in Gerbil Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Dietary Treatment Group</th>
<th>Phytoene (nmol/g)</th>
<th>Phytofluene (nmol/g)</th>
<th>cis-Lycopene (nmol/g)</th>
<th>all-trans Lycopene (nmol/g)</th>
<th>ζ-carotene (nmol/g)</th>
<th>β-carotene (nmol/g)</th>
<th>Total Lycopene (nmol/g)</th>
<th>Total Carotenoids (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>SO b</td>
<td>40.4 ± 9.6 a</td>
<td>21.9 ± 4.9 a</td>
<td>47.7 ± 8.7</td>
<td>51.2 ± 13.2</td>
<td>7.7 ± 2.1</td>
<td>3.2 ± 1.6</td>
<td>299.6 ± 113.2</td>
<td>372.7 ± 124.9</td>
</tr>
<tr>
<td></td>
<td>CO c</td>
<td>61.8 ± 11.0 b</td>
<td>39.6 ± 4.4 b</td>
<td>59.3 ± 10.7</td>
<td>39.5 ± 8.4</td>
<td>8.8 ± 2.2</td>
<td>1.6 ± 0.6</td>
<td>299.1 ± 96.7</td>
<td>410.8 ± 112.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>SO d</td>
<td>0.001 ± 0.0003</td>
<td>3.8 ± 0.6</td>
<td>17.8 ± 3.4</td>
<td>14.1 ± 2.5 a</td>
<td>0.52 ± 0.15</td>
<td>ND</td>
<td>22.2 ± 8.8 a</td>
<td>36.3 ± 19.3</td>
</tr>
<tr>
<td></td>
<td>CO e</td>
<td>0.001 ± 0.0003</td>
<td>5.1 ± 0.8</td>
<td>12.4 ± 2.1</td>
<td>0.73 ± 1.2 b</td>
<td>0.78 ± 0.17</td>
<td>ND</td>
<td>12.5 ± 4.3 b</td>
<td>25.6 ± 13.2</td>
</tr>
<tr>
<td>Adrenals</td>
<td>SO f</td>
<td>22.5 ± 8.2 a</td>
<td>10.2 ± 2.5</td>
<td>9.4 ± 2.4</td>
<td>5.4 ± 1.4</td>
<td>2.9 ± 7.7</td>
<td>ND</td>
<td>14.8 ± 9.8</td>
<td>50.4 ± 39</td>
</tr>
<tr>
<td></td>
<td>CO g</td>
<td>71.4 ± 17.9 b</td>
<td>18.3 ± 3.9</td>
<td>14.2 ± 3.1</td>
<td>6.9 ± 1.5</td>
<td>4.6 ± 1.1</td>
<td>ND</td>
<td>21.1 ± 12</td>
<td>115.5 ± 69.8</td>
</tr>
<tr>
<td>Lung</td>
<td>SO h</td>
<td>0.13 ± 0.01</td>
<td>0.16 ± 0.04 a</td>
<td>0.51 ± 0.17</td>
<td>0.28 ± 0.09</td>
<td>0.06 ± 0.01 a</td>
<td>ND</td>
<td>0.79 ± 0.8</td>
<td>1.1 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>CO i</td>
<td>0.45 ± 0.2</td>
<td>0.42 ± 0.2 b</td>
<td>0.99 ± 0.26</td>
<td>0.44 ± 0.12</td>
<td>0.17 ± 0.03 b</td>
<td>ND</td>
<td>1.4 ± 1.2</td>
<td>2.4 ± 1.8</td>
</tr>
<tr>
<td>Testes</td>
<td>SO j</td>
<td>0.2 ± 0.04 a</td>
<td>0.09 ± 0.02 a</td>
<td>0.17 ± 0.03 a</td>
<td>0.03 ± 0.006 a</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CO k</td>
<td>ND</td>
<td>0.65 ± 0.13 b</td>
<td>0.36 ± 0.08 b</td>
<td>0.57 ± 0.12 b</td>
<td>0.16 ± 0.03 b</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Prostate</td>
<td>SO l</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Seminal Vesicle Complex</td>
<td>CO m</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Serum</td>
<td>SO n</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CO o</td>
<td>0.03 ± 0.006</td>
<td>0.02 ± 0.004</td>
<td>0.04 ± 0.007 b</td>
<td>0.02 ± 0.01 b</td>
<td>0.02 ± 0.004 b</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Skin</td>
<td>SO p</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CO q</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Values are means ± the SEM, n=10. Different letters within rows indicate significant differences within tissues (p<0.05).

b SO = Safflower oil + 10% TP.
c CO = coconut oil + 10% TP.
d ND = non detectable within a tissue.
e NQ = not quantifiable within a tissue, but detected at very low levels.
f Units are μmol/dL.
Serum cholesterol concentrations of gerbils fed either a 20% safflower oil diet (SOC; n=10), 10% TP + 20% safflower oil diet (TPSO; n=10), 18% coconut oil diet + 2% safflower oil (COC; n=10), or 10% TP + 18% coconut oil diet + 25 safflower oil (TPCO; n=10), for 28 days. Significant differences between safflower and coconut oil diets (α=0.05), are denoted by letters. Columns represent the average of 10 analyses performed in triplicate and error bars represent ± SEM.
Figure 2.2. Hepatic Cholesterol Concentrations

Hepatic cholesterol concentrations of gerbils fed 20% safflower oil diet (n=10), 10% TP + 20% safflower oil diet (n=10), 18% coconut oil + 2% safflower oil diet (n=10), or 10% TP + 18% coconut oil + 2% safflower oil diet (n=10), for 28 days.

Significant differences between safflower and coconut oil diets (α=0.05), are denoted by letters.

Columns represent the average of 10 analyses performed in triplicate and error bars represent ± SEM.
REFERENCES


CHAPTER 3

A Low-Lycopene Containing Tomato Powder Diet Intervention Does Not Protect Against Prostate Cancer in the TRAMP Model

ABSTRACT

The Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model develops and progresses through all stages of carcinogenesis in a manner similar to humans. Our laboratory previously demonstrated a high lycopene tomato powder diet (TP) was effective in reducing carcinogenesis in the TRAMP model. The objective of the current study was to determine if a low-lycopene TP intervention impacted carcinogenesis at 3 time points. 8-week-old male C57BL/6 X FVB TRAMP mice were randomized to consume either an AIN-93G + 10% TP diet (N=90) or the AIN-93G control diet (N=88) and assigned to one of three sacrifice ages: 12 (N=59), 16 (N=60), or 20 (N=59) weeks of age. There was no difference between diets in overall cancer incidence at any time point. However, at 16 weeks of age, TP significantly increased high-grade PIN (HGP) (p=0.014) and significantly decreased poorly-differentiated (PD) (p=0.024) lesions compared to the control diet suggesting a modest reduction in cancer progression by TP at 16 weeks of age. Two variables that may explain the modest effect of TP in this study were: the amount of lycopene in the TP diet and the timing of the intervention. Although the TP diet had 30-fold less lycopene than our previous study, after 8 and 12 weeks of feeding, mice had accumulated similar tissue concentrations of carotenoids. In addition, the initiation of the diet intervention time of 8 weeks of age instead of 4 weeks of age, may have been too late in cancer progression to substantially impact carcinogenesis, as studies have shown prostate-specific gene expression changes as early as 8-10 weeks of age in TRAMP mice. This study further supports epidemiology data emphasizing lifelong consumption of tomatoes for PCa.
prevention and suggests that lycopene content of the TP may be critical in reducing PCa in this model.

1 Additional tables and figures not included in this chapter are in Appendix A.
INTRODUCTION

In 2014, prostate cancer (PCa) remained the second leading cause of cancer deaths in U.S. men. Due to increased screening and earlier detection of PCa, recommendations for localized PCas often include “watchful waiting” or “active surveillance” which involves frequent monitoring of the potential disease progression by digital rectal examination and monitoring of PSA levels in an attempt to avoid costly and unnecessary treatments with negative side effects. Men diagnosed with PCa often turn to dietary supplements as an inexpensive way to try and reduce the risk of cancer progression.

In 1995, Giovannucci et al. reported that the intake of tomato and tomato-based products was associated with a reduction in PCa risk. Lycopene, the predominant carotenoid found in tomatoes, has been widely investigated for its effects in PCa. Lycopene has been demonstrated to modulate growth factor signaling, cell-cycle programming, angiogenesis, and apoptosis in PCa cell lines. There have been numerous epidemiology studies examining the effects of lycopene and tomato products on PCa risk. Most recently, a high lycopene intake was shown to be inversely associated with total PCa and more strongly with lethal PCa in men taking part in the Health Professionals Follow-Up Study.

Results from animal studies examining lycopene, tomato powder (TP), and PCa have been promising. In 2003, Boileau et al. determined that consumption of a 10% TP diet reduced PCa incidence in N-methyl-N-nitrosurea (NMU)-testosterone-treated rats. Interestingly, they did not observe a protective effect in this model when lycopene was provided at the same dose in the beadlet form, suggesting that components from the whole tomato, in addition to lycopene, are important in preventing carcinogenesis. In the Dunning R3327-H rodent model of PCa, a 10% TP diet reduced tumor weight and volume compared to the control. Again, the TP diet reduced
tumor weight and volume to a greater extent when compared to diets supplemented with lycopene beadlets\textsuperscript{9}. In two studies using the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, a 10% TP diet resulted in a 39\% reduction in PCa incidence\textsuperscript{10} and increased overall survival compared to control diets\textsuperscript{11}. Collectively, these studies suggest that whole tomatoes may be more effective than lycopene alone in reducing prostate carcinogenesis.

Though \textit{in vivo} studies have produced promising results, they often model lifelong consumption of tomato products by beginning the TP diet after weaning. The use of complementary and alternative medicine (CAM) and changes in dietary patterns are common in cancer patients and in people suffering from chronic diseases\textsuperscript{2}. Butler et al., reported that in men diagnosed with PCa, 11\% reported using CAM beginning at the time of diagnosis for at least 6 months\textsuperscript{2}. Of those, 55\% of men reported using dietary supplements and 23\% reported dietary changes at diagnosis\textsuperscript{2}. Therefore, modeling lifelong consumption of lycopene intake may not accurately mimic the effects of dietary interventions men adopt following diagnosis of PCa.

To accurately model a dietary intervention, we initiated a 10\% TP diet in TRAMP mice at 8 weeks of age when histopathologically-confirmed prostatic intraepithelial neoplasia (PIN) lesions were present. Other studies began dietary interventions prior to initiation of carcinogenesis or puberty\textsuperscript{8–12}. The initiation of a TP diet in TRAMP mice after puberty when prostate-specific pathological changes occur has not been previously investigated. We hypothesized that a standard TP diet intervention directly after puberty would reduce PCa incidence in the TRAMP model.

\textbf{MATERIALS AND METHODS}

\textbf{Animal Methods.} The University of Illinois Laboratory Animal Care Advisory Committee approved all animal procedures. Female and male heterozygous TRAMP (C57BL/6)
mice from our colony were bred with FVB/NJ mice (Jackson Labs, CA) to obtain (TRAMPxFVB/NJ)F1 offspring for the study. Mice were genotyped via PCR-based DNA (Sigma-Aldrich, Saint Louis, MO) screening using established methods. Offspring were weaned at 3 weeks of age, individually housed in shoebox cages under controlled conditions (12 h light/dark cycle, 22 °C, 60% humidity) and acclimated to a pelleted, semi-purified AIN-93G diet for 4 weeks. At seven weeks of age, mice were acclimated to consume powdered AIN-93G control diet and at 8 weeks of age, mice were randomized to consume powdered study diets of AIN-93G control or AIN-93G + 10% TP until sacrifice. Sacrifice time points were randomized at weaning and mice were assigned to a sacrifice age of 12 weeks (n=29 control and n=30 TP), 16 weeks (n=30 control or n=30 TP), or 20 weeks of age (n=29 control and n=30 TP). Non-transgenic littermates were included in the study to confirm the effects of the transgene and sacrificed at 12 (n=5/diet), 16 (n=5/diet), and 20 weeks of age (n=14/diet). An additional cohort of mice (n=9) were fed control AIN-93G pellets and sacrificed at 8 weeks of age to confirm the stage of carcinogenesis we initiated our dietary intervention.

At 12, 16, and 20 weeks of age, mice were asphyxiated by CO2 and blood was collected via cardiac puncture. Serum was separated following centrifugation, aliquotted, frozen, and stored for analyses. The prostate was micro-dissected into individual lobes (anterior, ventral, and dorso-lateral). One half of each prostate lobe was fixed overnight in 10% phosphate-buffered formalin and then transferred to 70% ethanol for histological evaluation. All animals were thoroughly examined for gross metastases by trained research staff during the necropsy. A section of liver, lungs, and lymph nodes were fixed in 10% phosphate-buffered formalin and transferred to 70% ethanol after 24 hours for evaluation of micro-metastases. Liver, testes,
semenal vesicles, spleen, gonadal adipose, and one half of each prostate lobe were flash frozen in liquid nitrogen and stored at -80 °C.

**Diet Formulation.** Diets were balanced for protein, carbohydrates, fat, energy, and fiber and stored at 4 °C in the dark and have been previously used successfully in our lab\textsuperscript{8–10,13}. Carotenoid profiles of the diets were measured by high performance liquid chromatography and photodiode array (HPLC-PDA) Composition of experimental diets is shown in Table 3.1.

**Diet and Tissue Carotenoid Analyses.** Diet and tissue carotenoid analyses were performed using previously-published techniques described by our lab\textsuperscript{10,14,15}. Briefly, 5 mL of ethanol solution containing 0.01% BHT was added to tissue or 25 mg diet, 0.6 g gonadal adipose, or 0.1 g of liver. Anterior prostates were pooled within groups to facilitate HPLC detection of carotenoids. Both testes from the same animal were extracted as one replicate and 300 μL of serum was used for analyses. Tissue was minced thoroughly and diet was homogenized for 75 s in ethanol/BHT before addition of 1 mL saturated potassium hydroxide. Diet and tissues were saponified for 30 minutes at 60°C. Samples were extracted 3 times with 6 mL hexane, dried in a Speedvac (model AS160; Savant, Farmingdale, NY) evaporator, flushed with argon, and stored at −20 °C for ≤ 48 h before reverse-phase HPLC-PDA analysis as previously described\textsuperscript{15}. Analytical standards of lycopene, phytoene, phytofluene, and β-carotene were used for quantification. Samples were analyzed using HPLC-PDA (Waters, Milford, MA). A C30 reversed phase column (4.6 x 150 mm, 3 μm, YMC, Wilmington, NC) cooled to 18 °C was used with methyl-tert butyl ether, methanol, and 1.5% (w/v) aqueous ammonium acetate mobile phases to separate phytoene, phytofluene, lycopene, and β-carotene. Specific mobile phase composition and gradient elution method were used as described previously\textsuperscript{15}. Carotenoids were identified and quantified via UV spectra, retention times and standard comparison.
Lycopene (Sigma-Aldrich, Saint Louis, MO), phytoene, phytofluene, and ζ-carotene standards were purchased (Carotenature, Ostermundigen, Switzerland). β-carotene was extracted from 15% beadlets (DSM, Heerlen, Netherlands). Samples were analyzed within 48 hours of extraction. Samples were kept at 4 °C on an autosampler cooling tray and handled under yellow light to reduce carotenoid degradation.

**Histopathology.** One half of each lobe of the prostate (anterior, ventral, and dorso-lateral) was fixed in 10% formalin overnight and transferred to 70% ethanol. Cassettes were paraffin embedded, cut and stained with hematoxylin and eosin (H&E) before examination. A blinded and trained pathologist evaluated all of the lobes of the prostate and assigned a score for the most severe and most common lesion for each lobe according to a previously published grading scheme.¹⁶

**Serum Vascular Endothelial Growth Factor (VEGF).** Serum VEGF was measured using an ELISA kit (Abcam, San Francisco, CA) in mice at 12, 16 and 20 weeks of age.

**Real Time Quantitative PCR.** RNA was extracted from dorso-lateral prostate lobes using the RNeasy Mini Kit (Qiagen, Valencia, CA) and on-column DNase treated with an RNase-free DNase kit (Qiagen, Valencia, CA). RNA was reverse transcribed into complimentary DNA (cDNA) using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA). mRNA expression of selected genes was measured via real-time PCR using SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Reactions were monitored by an ABI Prism 7900HT. Prostatic levels of VEGF were determined using RT PCR validated primers (SA Biosciences, Valencia, CA). Prostatic levels of HSD17β2, HSD17β3, and 5-α reductase I were determined using RT PCR validated primers (Bio-Rad, Hercules, CA). RPL-19 was used as the housekeeping gene (SA Biosciences, Valencia, CA). Primer pairs were designed
for androgen receptor forward-5’-TCTTTCAAGGGAGGTACGC-3’ and reverse-5’-
AGGACGGGATCTCAAGTGTC-3’, CYP17A1 forward-5’- TAGGCTTCAGTCGAACACCG-
3’ and reverse-5’- TCCTTCGGATGGCAACTC-3’, β-microseminoprotein (msmb) forward-
5’-GTCAATCACCTGCTGATCAAC -3’ and reverse-5’-CTGGGTCTTCCGATCCAC-3’, 5-
α reductase II forward-5’-AAAGCCACTGCCTCTGCAT-3’ and reverse-5’-
AAAGCCACTGCCTCTGCAT-3’, and RPL-19 forward-5’-AAATCGCAATGCCAAGCTC-
3’ and reverse-5’-ACCCTTCCTTCCTATGC-3’ (IDT, Coralville, IA).

**Immunohistochemistry.** Immunohistochemistry for proliferating cell nuclear antigen
(PCNA) and cleaved caspase-3 (CC3) were performed on paraffin-embedded (4 µm) fixed
sections of the dorso-lateral prostate lobe according to a previously published protocols.17,18
Briefly, slides were placed in a decloaking chamber and treated in a citrate buffer (pH 6.0) for 30
seconds at 125 ºC and 10 seconds at 90 ºC for antigen retrieval. In a BioGenex i6000 Automated
Staining System, (BioGenex, San Ramon, CA), endogenous peroxide was quenched with a 3%
H₂O₂ solution for 15 minutes, slides were blocked with Power Block™ (BioGenex) for 10
minutes, avidin blocked for 15 minutes, and then incubated with rabbit ant-proliferating cell
nuclear antigen (PCNA) antibody (Abcam, Cambridge, MA) or rabbit anti-cleaved caspase-3
(Cell Signaling, Danvers, MA) for 30 minutes and visualized using a SuperSensitive™ Link-
Label IHC Detection System (BioGenex). Slides were stained with DAB and counterstained with
hematoxylin. Mouse small intestinal tissue was used as the positive control for PCNA and mouse
thymus tissue was used as the positive control for cleaved caspase-3. Negative controls were
generated by omitting the primary antibody. Stained slides were scanned with a NanoZoomer
2.0-HT digital slide scanner (Hamamatsu, Bridgewater, NJ) with Olympus Uplansapo 20X
objective at 40X digital zoom, resulting in 0.23 µm resolution. Images were captured with NDP
view software (Hamamatsu). One image from the dorso-lateral lobe was blindly quantified and the proliferative index (PI) percentage (PCNA positive-stained nuclei/ total nuclei counted x 100) and apoptotic index (AI) percentage (number of activated caspase-3 cells/ total nuclei counted x 100) calculated. These indices were established by counting at least 1000 cells from each image.

**Ultrasound Imaging.** TRAMP mice were imaged at 10, 12, 16, 18, and 20 weeks of age. Mice were anesthetized prior to ultrasound imaging using 2% isofluorane, abdominal fur was trimmed, and mice were placed on a heated stage for imaging. 2-dimensional (2-D), 3-dimensional (3-D), and Doppler ultrasound images were acquired by a trained ultrasonographer using the VisualSonics Vevo 2100 high frequency imaging system (VisualSonics, Toronto, Canada). 3-D images were constructed from 2-D images taken at special intervals using the Vevo 2100 software. Volume was calculated from the constructed 3-D image by manual tracing. Each prostate was traced three times and the volumes were averaged to minimize tracing error.

**Serum Testosterone.** Serum testosterone was extracted from 150 µL of serum using diethyl ether. After the ether evaporated, a hexane/methanol extraction was used to extract the testosterone from other serum lipids for analyses. Serum testosterone was evaluated using the Coat-A-Count Total Testosterone Radioimmunoassay (Siemens TKTT2, Los Angeles, CA).

**Statistical Analyses.** Data are shown as means ± SEM. Carotenoid analyses, serum VEGF, serum testosterone, and proliferation and apoptosis indexes were compared among treatments by one-way analysis of variance (ANOVA) followed by post-hoc Tukey-Kramers studentized range test with α=0.05 when the assumptions of ANOVA were met; otherwise the Wilcoxon and Kruskal-Wallis non-parametric test was used. AR, HSD17β2, HSD17β3, 5α-reductase I, 5α-reductase II, VEGF, and CYP17A1 were first logarithmically transformed to
meet the assumptions of ANOVA. Msmb PCR data was not transformed. All of the mRNA expression data was analyzed using 2-tailed ANOVA followed by post-hoc Tukey-Kramers studentized range test with $\alpha=0.05$. Ultrasound volumes were analyzed using ANOVA with repeated measures and compared using Tukey-Kramers studentized range test. Additionally, linear regression was performed using the REG procedure in SAS. Fisher’s exact test was used to compare histopathology scores between the TP group and the control group and each age was analyzed separately. Differences in overall cancer incidence, tumor incidence, and metastases incidence were analyzed using Fisher’s exact test. All statistical analyses were conducted with SAS (version 9.2; SAS Institute, Cary, NC, USA).

RESULTS

**Body Weight and Food Intake.** All diets were readily consumed and there were no significant differences in food intake between diets or between groups ($p<0.05$). Final body weights were significantly higher in TP-fed ($p=0.02$) than control-fed mice only at 16 weeks of age. All other beginning and final body weights and intakes were not statistically different between groups (Table 3.2). Organ weights are listed as a percent of body weight in Table 3.3. In TRAMP mice at 16 weeks of age, control-fed mice had significantly increased urogenital tract (UGT) weight compared to TP-fed mice ($p=0.005$). Furthermore, TP-fed mice had significantly increased gonadal adipose ($p=0.002$) compared to control-fed mice. Lastly, TP-fed wild-type mice at 16 weeks of age had increased liver weight ($p=0.005$) as a percent of body weight compared to control wild-type mice.

**Diet Carotenoid Profiles.** Diets were prepared in five batches over the duration of the study. There was no difference between carotenoid content between each batch. The most abundant carotenoid in the diet used in this study was phytoene, a colorless precursor to lycopene.
(Figure 3.1). Lycopene was only present at 23 µmol/Kg (12.3 ppm) diet, which is substantially lower than previous TP studies conducted in our lab\textsuperscript{9,10,13}. The TP sourced for the current study was provided by Futureceuticals (Momence, IL) and is a standard drum-dried TP with the seeds and skins included. This lot of TP had considerably lower levels of tomato carotenoids compared to the 286 ppm TP used by Zuniga et al. and other studies carried out in our lab\textsuperscript{10,19,20}.

**Tissue Carotenoid Accumulation.** Liver, gonadal adipose, testes, serum, and prostatic carotenoid accumulation is shown in Table 3.4. Mirroring the diet (see Figure 3.1), hepatic lycopene was lower than hepatic phytoene at all ages. Additionally, total gonadal adipose carotenoid accumulation was significantly increased in mice at 20 weeks of age when compared to mice at 12 weeks of age (p=0.02). Lycopene deposition increased significantly in gonadal adipose (p=0.01) in mice at 20 weeks of age compared to mice at 12 weeks of age. As previously observed in prostate and other androgen-sensitive tissues, phytoene was absent from the prostate and testes\textsuperscript{15,19}.

**Histopathology.** We confirmed through histopathological evaluation of 8 week-old TRAMP mice that they had moderate- and high-grade PIN lesions when our dietary intervention was initiated. This confirms that our dietary intervention was initiated when the prostate was in a precancerous stage and was no longer considered normal tissue. There were no differences due to diet in the most severe or most common lesion in either the anterior, ventral, and dorso-lateral lobes at 12, 16 or 20 weeks of age. Furthermore, there were no significant differences when all lobes were combined for the most severe and most common lesion overall at 12, 16, or 20 weeks of age. Table 3.5 shows the distribution of scores between diets at each age. When examined by cancer stage within each age, there were no differences in the 12 or 20 week old animals. However, mice at 16 weeks of age consuming the TP diet had significantly increased high-grade
PIN (HGP) lesions (p=0.014) and significantly decreased incidence of poorly differentiated (PD) lesions (p=0.024) than mice on the control diet. Although total cancer incidence between tomato and control diets at 16 weeks of age was not different, this may suggest that the TP diet modestly delayed the progression from HGP to PD lesions at this age. The diet effect was not observed in mice at 20 weeks of age suggesting that the transition from precancerous to cancerous lesions might be short-lived or overwhelmed by the aggressive growth of early developing PD tumors.

The incidences of gross prostate tumors and gross metastases to pelvic lymph nodes were examined at necropsy and the results are represented in Table 3.6. There were no gross tumors or metastases observed in 12 week old TRAMP mice. However, in 16 week old mice, there was a statistically significant increase (p=0.05) in the incidence of gross prostate tumors in control-fed mice compared to the TP-fed mice. Similarly to the prostate histopathological data, there was no difference in the incidence of gross prostate tumors and gross pelvic lymph node metastases in mice at 20 weeks of age.

**Serum VEGF.** Serum VEGF was significantly increased (p=0.001) in mice at 20 weeks of age compared to mice at 12 and 16 weeks of age (Figure 3.2). No significant differences were observed between diets at any age (Figure 3.2), but TP-fed mice had numerically less amount of VEGF at 12, 16, and 20 weeks of age.

**VEGF-A mRNA Expression.** Prostatic expression of VEGF-A is represented in Figure 3.3. Similar to serum VEGF levels, there was no difference between diets at 16 and 20 weeks of age, but TP tended to reduce VEGF-A expression levels at 16 weeks of age, though this was not statistically significant. In contrast, there was a trend that prostatic VEGF-A was increased by TP at 20 weeks, though non-significantly.
**Immunohistochemistry.** The apoptotic index (AI) of prostates and tumors stained positive for CC3 is represented in Figure 3.4. There was no difference in AI between diets or between ages. Tumors had significantly increased AI than prostates with HGP \( (p < 0.0001) \). The proliferation index (PI) is represented in Figure 3.5. There were no differences in PI between diets in mice with HGP.

**Serum Testosterone.** We measured serum testosterone in TRAMP mice fed either a 10% TP containing diet or a control diet at 12, 16, and 20 weeks of age (Table A.1). At all ages, the TP diet numerically increased levels of serum testosterone. Serum testosterone was significantly increased in mice fed the TP diet at 16 \( (p=0.05) \) and 20 weeks of age \( (p=0.02) \) when compared to the control diet. Interestingly, serum testosterone was numerically, but not significantly, decreased in mice with tumors than in mice with HGP. (Appendix A).

**Androgen Receptor mRNA Expression.** In the current study, TP-fed mice had significantly \( (p=0.01) \) increased expression of AR at 20 weeks of age compared to control fed mice (Figure A.2). Furthermore, tumoral expression of AR was significantly lower than in mice with HGP \( (p=0.001) \), though no significant differences due to diet were detected (Figure A.3, Appendix A).

**5-alpha reductase I and II Expression.** Prostatic mRNA expression of 5α-reductase I and II were not statistically different between control and TP groups at 12, 16, or 20 weeks of age. However, there was a significant decrease in mRNA expression of 5α-reductase I \( (p=0.02) \) and II \( (p<0.001) \) in mice with tumors compared to mice with PIN (Figure A.4, Appendix A).

**CYP17A1 mRNA Expression.** There was no difference in CYP17A1 expression between diet groups at 12, 16, or 20 weeks of age. There was a significant increase in CYP17A1 expression in tumors than in HGP \( (p < 0.001) \) (Figure A.5, Appendix A).
HSD17β2 and HSD17β3 mRNA Expression. mRNA expression of HSD17β2 and HSD17β3 was not different between diets at 12, 16, and 20 weeks of age. In mice with tumors, HSD17β3 expression was significantly decreased (p=0.004) compared to mice with HGP (Figure A.6, Appendix A). There was no significant pathology effect of HSD17β2.

β-microseminoprotein (msmb) mRNA Expression. Prostatic msmb signaling was significantly decreased (p<0.001) in mice with tumors compared to mice with PIN (Figure A.7, Appendix A). There was no effect of diet at 12, 16, or 20 weeks of age.

Ultrasound Imaging. Longitudinal 3-D images of the prostate were taken every two weeks. 3-D ultrasound volumes of tumors were highly correlated with tumor volume and mass at necropsy. 3-D calculated volume at 20 weeks of age was correlated at 0.98 with gross tumor mass (Figure A.8) and 0.98 with gross tumor volume as measured by calipers (Figure A.9). In the current study, there was no difference in the onset or rate of growth of tumors between TP (n=5) and control-fed (n=6) mice. As noted before, not all TRAMP mice who underwent ultrasound imaging developed a prostate tumor, therefore we statistically analyzed mice with tumors separately from mice without tumors. Longitudinal volumes of mice with tumors were not different between groups (Figure A.10, Appendix A). Volumes of mice without tumors were not significantly different between groups (Figure A.11, Appendix A).

DISCUSSION

There are a number of rodent studies examining the effect of lifelong (post-weaning) TP consumption on PCa. Previously, we and others observed a reduction in PCa by including a high-lycopene containing 10% TP in the diets of TRAMP mice. In the current study, we hypothesized that a 10% standard TP diet initiated at 8 weeks of age when preneoplastic prostatic lesions were present, would reduce the incidence of PCa compared to the control diet at
12, 16, and 20 weeks of age. However, total cancer incidence, as defined as having at least one of the following lesions: well differentiated (WD), moderately differentiated (MD), or PD, was not statistically impacted by diet at either 12, 16, or 20 weeks of age.

Despite the lack of a significant decrease in overall cancer incidence, TP may have been effective in delaying the progression from precancerous lesions to cancerous lesions in mice at 16 weeks of age. TP-fed mice at 16 weeks of age had a significantly increased incidence of HGP and a significantly decreased incidence of PD lesions compared to control-fed mice (p=0.01). This suggests that TP may have delayed the progression from precancerous HGP lesions to invasive PD lesions at this single time point. Additionally, 16 week old control-fed mice had increased urogenital-tract weight (p=0.005) and an increased incidence of gross tumors at necropsy than TP-fed mice. This may suggest that control-fed mice had more advanced tumors that were larger at necropsy and may have had a more invasive potential than TP-fed mice. However, any differences in pathologies were lost by 20 weeks of age.

Angiogenesis is a process by which new vasculature can be formed. Men with PCa have been shown to have increased serum levels and expression of VEGF\textsuperscript{11,21}. We measured serum levels of VEGF at 12, 16, and 20 weeks of age and prostatic mRNA expression of VEGF-A in mice at 16 and 20 weeks of age to determine if a 10% TP diet intervention reduced VEGF-A and by inferences, angiogenesis, compared to the control mice. While mice at 20 weeks of age had significantly increased levels of serum VEGF, there were no significant differences between diets at any age. However, there was a slight trend that serum VEGF was numerically reduced by TP at 12, 16, and 20 weeks of age. Prostatic mRNA expression of VEGF followed a similar trend as serum at 16 weeks of age, in that it appeared there was a numerical reduction of VEGF by the TP diet, however, that was not the case at 20 weeks of age.
Apoptosis and proliferation have been shown to be impacted by TP and lycopene in cell and animal models of PCa\textsuperscript{5,9,10,22,23}. However, in the current study there were no significant differences in the AI and PI between dietary groups at 12, 16, and 20 weeks of age. There was a trend toward reduced apoptosis in the TP group at 16 weeks of age, though the change was non-significant. There were no diet effects on prostatic gene expression. However, there were significant differences between expression in prostates with HGP and in prostate tumors. Tumors had significantly reduced expression of AR suggesting that they relied on a different source of androgens for growth and development and possibly suggesting a castration-resistant PCa phenotype. CYP17A1 and 5-alpha reductase II expression in tumors also indicated a shift towards a castration-resistant PCa phenotype. However, tumors had significantly lower expression of HSD17β3 which is typically over-expressed in advanced PCa.

While we observed a modest effect on cancer incidence by TP at 16 weeks of age, we suggest that there are two variables that may have contributed to the lack of a robust effect in overall cancer incidence: 1) the carotenoid content of the TP and 2) the timing of our intervention.

In previous studies using rodent models of PCa, TP and/or lycopene have been observed to drastically reduce PCa incidence\textsuperscript{8–10,24}. However, the current study resulted in a modest reduction. A commercially-available drum-dried TP including the skins and seeds was analyzed and used to formulate our intervention diet. The TP used was a standard TP containing more than 30-fold less lycopene than the TP Zuniga et al. used\textsuperscript{13}. There are several factors that can influence tomato carotenoid content, including geographical location grown and growing season, cultivation methods, weather conditions, use of nitrogenous fertilizers, and the tomato variety\textsuperscript{25,26}.
The lower lycopene content of the TP used in the current study could well be responsible for the modest results observed. Previously, TP diets used in TRAMP studies provided lycopene ranging from 132-286 ppm, and were effective at reducing PCa incidence\textsuperscript{10,11}. Zuniga et al. observed a 39\% decrease in cancer incidence in mice consuming 286 ppm lycopene from a 10\% TP diet\textsuperscript{10}. In another study, increased overall survival of TRAMP mice was observed in mice consuming 132 ppm lycopene from a 10\% TP diet\textsuperscript{24}. However, Konijeti et al., determined that 28 ppm lycopene from a tomato paste diet was ineffective in reducing PCa incidence in the TRAMP model\textsuperscript{27}. Interestingly, 100 ppm of lycopene from beadlets was effective in that study\textsuperscript{27} The current study provided only 12.3 ppm lycopene from the 10\% TP diet. Collectively, the results of these TRAMP studies suggest a possible threshold level of lycopene necessary to affect cancer incidence in this aggressive transgenic model of PCa. It is interesting to note, however, that levels of carotenoids found in the gonadal adipose, serum, and prostate were similar to a previous study in our laboratory, whereas, hepatic lycopene at 20 weeks of age was four-fold lower than previously observed at 18 weeks of age (Figure 3.6/Table 3.7)\textsuperscript{10}.

An alternative hypothesis for the findings is related to the timing of the dietary intervention. The TP intervention was designed to mimic the dietary changes a man newly diagnosed with PCa might adopt. In our study, TP diets were consumed post-puberty when prostates had already progressed to low- and moderate-grade PIN. It is possible that the dietary intervention was too late to delay or impact the transgenic cancer process in this aggressive model of PCa. Research has shown that by 10 weeks of age, there is a genotype effect in TRAMP mice that results in an up-regulation of genes involved in cell growth and cell-cycle regulation\textsuperscript{12}. A 10\% TP diet initiated at 4 weeks of age in TRAMP mice was also shown to alter androgen metabolism and signaling at 10 weeks of age\textsuperscript{12}. This may suggest that there is a critical
period of time where dietary studies of TP and lycopene may be effective in this model. Other studies utilizing TP and/or lycopene in the TRAMP model began dietary treatments at 3 and 4 weeks of age, after weaning and before puberty, and observed significant effects in cancer outcomes by diet\textsuperscript{10,11}. Our study suggests that beginning a dietary intervention after puberty results in a less robust effect in cancer incidence in this model. Additionally, while carotenoid accumulation in tissues was similar between studies at 16 and 20 weeks of age, tissues at 12 weeks of age had lower amounts of carotenoid concentrations (Table 3.4), indicating that four weeks of TP feeding may not be enough to significantly alter genes involved in androgen metabolism and synthesis to impact this critical time in cancer development. Epidemiological evidence suggests that long-term or early dietary lycopene intake is likely more effective for PCa prevention than recent intake\textsuperscript{30}. Together, this study further emphasizes the importance of lifelong consumption of tomatoes and/or lycopene-containing foods to potentially reduce the risk of PCa.

According to the USDA National Nutrient Database values for lycopene in tomato products, the current study diet provided the amount of lycopene found in 1.4 cups of raw cherry tomatoes; an amount realistically consumable by an adult. Although total cancer incidence was not different between diets, TP may have delayed the progression of preneoplastic PCa to invasive PCa at 16 weeks of age. There may be a critical period of lycopene exposure necessary to confer protection in PCa and future studies should continue to determine if early lycopene exposure is key in delaying the development of PCa. It is clear that tomatoes contain components that alter androgen signaling in the prostate, however, the minimum amount required to observe an effect appears to vary between animal models. Future studies examining TP should focus on
the effect of exposure timing and lycopene content of the diet to determine the effectiveness of this dietary approach for reducing the risk of PCa.

**ABBREVIATIONS USED**

BHT, butylated hydroxytoluene; HPLC-PDA, high performance liquid chromatography-photodiode array.

**ACKNOWLEDGMENTS**

The authors would like to thank FutureCeuticals for their generous donation of TP for this study.
### FIGURES AND TABLES

**Table 3.1. Composition of Experimental Diets**

<table>
<thead>
<tr>
<th>g/100g total diet</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN-93G Control</td>
<td>AIN-93G + 10% TP&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Cornstarch</strong></td>
<td>39.7</td>
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</tr>
<tr>
<td><strong>Casein</strong></td>
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<td>18.5</td>
</tr>
<tr>
<td><strong>Maltodextrin</strong></td>
<td>13.2</td>
<td>11.8</td>
</tr>
<tr>
<td><strong>Sucrose</strong></td>
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<td>10</td>
</tr>
<tr>
<td><strong>Fiber&lt;sup&gt;b&lt;/sup&gt;</strong></td>
<td>5</td>
<td>3.3</td>
</tr>
<tr>
<td><strong>Mineral Mix&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Vitamin Mix&lt;sup&gt;d&lt;/sup&gt;</strong></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>L-Cystine</strong></td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Choline Bitrate</strong></td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Soybean Oil</strong></td>
<td>7</td>
<td>6.5</td>
</tr>
<tr>
<td><strong>Tomato Powder&lt;sup&gt;e&lt;/sup&gt;</strong></td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> 10% TP contains 23.8 μmol of lycopene, 30.1 μmol of phytoene, 16.7 μmol of phytofluene, and 2.6 μmol of β-carotene per Kg of diet.

<sup>b</sup> Non-nutritive cellulose

<sup>c</sup> AIN-93G-MX formation

<sup>d</sup> AIN-93G-VX formation

<sup>e</sup> FutureCeuticals Drum Dried Tomato Powder-20
Table 3.2. TRAMP and Wild-Type Body Weights and Food Intake

<table>
<thead>
<tr>
<th>Age</th>
<th>TRAMP</th>
<th>Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AIN-93G Control</td>
<td>10% Tomato Powder</td>
</tr>
<tr>
<td></td>
<td>Beginning Body Weight</td>
<td>Ending Body Weight</td>
</tr>
<tr>
<td>12 weeks of age</td>
<td>19.3 ± 0.6 21 ± 0.6</td>
<td>21.4 ± 0.7 18 ± 1.8</td>
</tr>
<tr>
<td>16 weeks of age</td>
<td>19.2 ± 0.5 19.5 ± 0.4</td>
<td>19.6 ± 0.5 20 ± 0.9</td>
</tr>
<tr>
<td>20 weeks of age</td>
<td>19.8 ± 0.6 18 ± 0.5</td>
<td>20.4 ± 1 18.7 ± 0.6</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM.

Different superscript letters within a row and within the same genotype indicate significant differences between diets (p<0.05).
### Table 3.3. TRAMP and Wild-Type Organ Weights as a Percent of Final Body Weight

<table>
<thead>
<tr>
<th>TRAMP</th>
<th>UGT</th>
<th>Liver</th>
<th>Seminal Vesicles</th>
<th>Testes</th>
<th>Gonadal Adipose</th>
<th>Lungs</th>
<th>Spleen</th>
<th>Ventral Prostate</th>
<th>Anterior Prostate</th>
<th>Dorso-lateral Prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 weeks</td>
<td>Control</td>
<td>1.6 ± 0.3</td>
<td>4.1 ± 0.8</td>
<td>1.1 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>3.6 ± 0.7</td>
<td>0.8 ± 0.2</td>
<td>0.3 ± 0.05</td>
<td>0.067 ± 0.01</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Tomato</td>
<td>1.7 ± 0.32</td>
<td>4.2 ± 0.8</td>
<td>1.2 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>3.8 ± 0.7</td>
<td>0.8 ± 0.2</td>
<td>0.3 ± 0.05</td>
<td>0.06 ± 0.01</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>16 weeks</td>
<td>Control</td>
<td>3.7 ± 0.7</td>
<td>3.9 ± 0.7</td>
<td>1.2 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>4.1 ± 0.8</td>
<td>0.7 ± 0.1</td>
<td>0.2 ± 0.06</td>
<td>0.09 ± 0.02</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Tomato</td>
<td>2.1 ± 0.4$^a$</td>
<td>3.9 ± 0.8</td>
<td>1.2 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>4.9 ± 0.9$^a$</td>
<td>0.7 ± 0.1</td>
<td>0.2 ± 0.05</td>
<td>0.08 ± 0.02</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>20 weeks</td>
<td>Control</td>
<td>7.5 ± 0.71</td>
<td>4 ± 0.7</td>
<td>1.4 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>3.6 ± 0.8</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.06</td>
<td>0.1 ± 0.02</td>
<td>0.2 ± 0.04</td>
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<tr>
<td></td>
<td>Tomato</td>
<td>7.5 ± 1.4</td>
<td>3.9 ± 0.7</td>
<td>1.6 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>3.6 ± 0.7</td>
<td>0.7 ± 0.1</td>
<td>0.3 ± 0.07</td>
<td>0.1 ± 0.02</td>
<td>0.2 ± 0.04</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Wild Type</th>
<th>UGT</th>
<th>Liver</th>
<th>Seminal Vesicles</th>
<th>Testes</th>
<th>Gonadal Adipose</th>
<th>Lungs</th>
<th>Spleen</th>
<th>Ventral Prostate</th>
<th>Anterior Prostate</th>
<th>Dorso-lateral Prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 weeks</td>
<td>Control</td>
<td>1.6 ± 0.9</td>
<td>4.3 ± 2.1</td>
<td>1 ± 0.5</td>
<td>0.7 ± 0.4</td>
<td>5 ± 2.5</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.05 ± 0.02</td>
<td>0.02 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Tomato</td>
<td>1.3 ± 0.7</td>
<td>4.6 ± 2.6</td>
<td>0.8 ± 0.5</td>
<td>0.7 ± 0.4</td>
<td>4.1 ± 2.4</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.03 ± 0.02</td>
<td>0.1 ± 0.08</td>
</tr>
<tr>
<td>16 weeks</td>
<td>Control</td>
<td>1.1 ± 0.5</td>
<td>3.9 ± 1.9</td>
<td>0.7 ± 0.4</td>
<td>0.5 ± 0.3</td>
<td>4.6 ± 2.3</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.03 ± 0.02</td>
<td>0.1 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Tomato</td>
<td>1.1 ± 0.6</td>
<td>4.3 ± 2.1$^a$</td>
<td>1 ± 0.5</td>
<td>0.5 ± 0.2</td>
<td>5 ± 2.5</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.03 ± 0.02</td>
<td>0.1 ± 0.06</td>
</tr>
<tr>
<td>20 weeks</td>
<td>Control</td>
<td>1.3 ± 0.4</td>
<td>4.1 ± 1.2</td>
<td>0.9 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>3.7 ± 1.1</td>
<td>0.2 ± 0.01</td>
<td>0.2 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.1 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Tomato</td>
<td>1.3 ± 0.4</td>
<td>4 ± 1.2</td>
<td>0.9 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>4.4 ± 1.3</td>
<td>0.2 ± 0.09</td>
<td>0.2 ± 0.09</td>
<td>0.04 ± 0.01</td>
<td>0.1 ± 0.04</td>
</tr>
</tbody>
</table>

Values represent means ± SEM.

# indicates a significant difference (p<0.05) from the control group within a tissue and within an age.
Figure 3.1. Percentage of Carotenoids in a 10% TP Diet

Percentage of Carotenoids in 10% Tomato Powder Diet

- Phytoene: 42%
- Phytofluene: 32%
- Lycopene: 23%
- Beta-carotene: 4%
Table 3.4. Serum and Tissue Carotenoid Concentration in 10% TP-fed TRAMP

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Serum (umol/L) (n=5)</th>
<th>Liver (nmol/g) (n=12)</th>
<th>Gonadal Adipose (nmol/g) (n=10)</th>
<th>Testes (nmol/g) (n=10)</th>
<th>Anterior Prostate (nmol/g) (n=6-8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 weeks</td>
<td>16 weeks</td>
<td>20 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytoene</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lycopene</td>
<td>0.47 ± 0.07</td>
<td>0.81 ± 0.18</td>
<td>0.66 ± 0.15</td>
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</tr>
<tr>
<td>β-carotene</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Total Carotenoids</td>
<td>0.47 ± 0.07</td>
<td>0.81 ± 0.18</td>
<td>0.66 ± 0.15</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Phytoene</td>
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<td>3.1 ± 0.2</td>
<td>3.2 ± 0.8</td>
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<td></td>
</tr>
<tr>
<td>Phytofluene</td>
<td>2.1 ± 0.5</td>
<td>2.6 ± 0.8</td>
<td>1.3 ± 0.4</td>
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<tr>
<td>Lycopene</td>
<td>1.4 ± 0.2</td>
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<tr>
<td>Total Carotenoids</td>
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<td>8 ± 1.2</td>
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<tr>
<td>Phytoene</td>
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<td>0.07 ± 0.02</td>
<td>0.07 ± 0.03</td>
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<tr>
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<td>0.03 ± 0.005</td>
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<tr>
<td>β-carotene</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Total Carotenoids</td>
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<td>0.4 ± 0.06 ab</td>
<td>0.6 ± 0.2 b</td>
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<tr>
<td>Lycopene</td>
<td>0.3 ± 0.05</td>
<td>0.4 ± 0.07</td>
<td>0.4 ± 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-carotene</td>
<td>ND</td>
<td>ND</td>
<td>0.1 ± 0.01</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Total Carotenoids</td>
<td>0.03 ± 0.04</td>
<td>0.4 ± 0.06</td>
<td>0.4 ± 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytoene</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>0.4 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lycopene</td>
<td>0.4 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-carotene</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Total Carotenoids</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are the mean ± SEM.

Different superscript letters within a row indicate significant differences between ages (p<0.05).

ND = carotenoid levels below detection limit of detection (0.02 nmol/g phytoene, 0.5 nmol/g phytofluene, and 0.5 nmol/g β-carotene).
Table 3.5. TRAMP Histopathology Results as a Percentage of Total Prostatic Lesions by Age and Diet

<table>
<thead>
<tr>
<th>TRAMP</th>
<th>PIN</th>
<th>Age (weeks)</th>
<th>NP</th>
<th>LGP</th>
<th>MGP</th>
<th>HGP</th>
<th>PLL</th>
<th>WD</th>
<th>MD</th>
<th>PD</th>
<th>Prostate Cancer (WD-PD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28 12 3.6% 0% 0% 89.3% 3.6% 0% 3.6% 0% 89.3% 3.6%</td>
</tr>
<tr>
<td>Tomato</td>
<td>30</td>
<td>12</td>
<td>6.7%</td>
<td>3.3%</td>
<td>3.3%</td>
<td>80%</td>
<td>0%</td>
<td>0%</td>
<td>6.7%</td>
<td>0%</td>
<td>6.7%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tomato Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 16 6.7% 0% 10% 43.3% 6.7% 6.7% 10% 16.7% 33.3%</td>
</tr>
<tr>
<td>Tomato</td>
<td>30</td>
<td>16</td>
<td>0%</td>
<td>0%</td>
<td>3.3%</td>
<td>76.7%#</td>
<td>3.3%</td>
<td>6.7%</td>
<td>10%</td>
<td>0%</td>
<td>6.7%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tomato Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>29 20 3.4% 0% 3.4% 31% 10.3% 3.4% 27.6% 20.7% 51.7%</td>
</tr>
<tr>
<td>Tomato</td>
<td>30</td>
<td>20</td>
<td>3.3%</td>
<td>0%</td>
<td>3.3%</td>
<td>40%</td>
<td>6.7%</td>
<td>13.3%</td>
<td>10%</td>
<td>26.7%</td>
<td>50%</td>
</tr>
</tbody>
</table>

NP = Normal Prostate, LG = low grade PIN, MG = moderate grade PIN, HG = high-grade PIN, PLL = phyllode-like lesions, WD = well differentiated, MD = moderately differentiated, PD = poorly differentiated.

Results are the incidence of each stage of pathology and overall incidence (sum of WD-PD) within dietary groups.

# Indicates a significant difference (p<0.05) from the control group at 16 weeks of age.
Table 3.6. TRAMP Gross Tumor and Lymph Node Metastases Incidence by Diet and Age

<table>
<thead>
<tr>
<th>Weeks of Age</th>
<th>n</th>
<th>Diet</th>
<th>Tumor</th>
<th>Lymph Node Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>29</td>
<td>Control</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Tomato</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>16</td>
<td>30</td>
<td>Control</td>
<td>33%</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Tomato</td>
<td>10% #</td>
<td>0%</td>
</tr>
<tr>
<td>20</td>
<td>29</td>
<td>Control</td>
<td>45%</td>
<td>24%</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Tomato</td>
<td>37%</td>
<td>20%</td>
</tr>
</tbody>
</table>

Values represent the percentage of TRAMP mice with gross tumors and gross pelvic lymph node metastases by age and diet.

# Indicates a significant difference (p<0.05) from the control at 16 weeks of age.
**Figure 3.2. TRAMP Serum VEGF by Age and Diet**

Values are the mean ± SEM.

# Indicates a significant difference (p<0.05).

**Figure 3.3. Prostatic mRNA Expression of VEGF-A by Age and Diet**

Values are the mean ± SEM.
**Figure 3.4. Prostatic Apoptosis Index at 16 Weeks of Age**

![Prostatic Apoptosis Index at 16 Weeks of Age](image1.png)

Values are the mean ± SEM.

**Figure 3.5. Prostatic Proliferation Index at 16 Weeks of Age**

![Prostatic Proliferation Index at 16 Weeks of Age](image2.png)

Values are the mean ± SEM.
Figure 3.6. Tissue Lycopene Accumulation between Studies

![Lycopene Tissue Accumulation](image)

Values are the mean ± SEM.

Current study tissue carotenoid concentrations were measured at 20 weeks of age. Zuniga et al.\textsuperscript{10} tissue carotenoid concentrations were measured at 18 weeks of age.

Table 3.7. Tissue Lycopene Accumulation between Studies

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Current Study</th>
<th>Zuniga et al.\textsuperscript{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 weeks</td>
<td>18 weeks</td>
</tr>
<tr>
<td>Serum (µmol/L)</td>
<td>0.66 ± 0.15</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td>Liver (nmol/g)</td>
<td>1.9 ± 0.3</td>
<td>4.56 ± 0.91</td>
</tr>
<tr>
<td>Gonadal Adipose (nmol/g)</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.19</td>
</tr>
<tr>
<td>Testes (nmol/g)</td>
<td>0.4 ± 0.03</td>
<td>1.42 ± 0.23</td>
</tr>
<tr>
<td>Prostate (nmol/g)</td>
<td>0.4 ± 0.1</td>
<td>0.25 ± 0.05</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM.
REFERENCES


CHAPTER 4

Pelleting Diets Impairs TRAMP Prostate Carcinogenesis

ABSTRACT

Prostate cancer (PCa) is the most commonly diagnosed male cancer in U.S. men. Daidzein, the major isoflavone present in soy germ, can be metabolized by the gut microbiota into equol. The effects of daidzein and equol on PCa are not well studied. The objective of this study was to investigate the effect of feeding 2% soy germ, daidzein, or equol on the progression of PCa in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. 3-week old male C57BL/6 X FVB TRAMP mice were weaned from our breeding colony and immediately acclimated to an AIN-93G control diet for one week. At 4 weeks of age, mice (n=30 per diet group) were randomized to one of four pelleted study diets, AIN-93G control, AIN-93G + 2% soy germ, AIN-93G + 92 ppm daidzein, or AIN-93G + 88 ppm equol until 18 weeks of age. To our surprise, we did not detect any statistical differences in cancer incidence between diets. Previously, we observed a 100% cancer incidence in TRAMP mice fed a control diet until 18 weeks of age, however, the control group in this study only had a 24% incidence in cancer at this same age. These findings are likely the result of the physical attributes of the pelleted study diets. Mice fed pelleted diets had lower food intake and significantly decreased body weights (p<0.001) compared to those fed powdered diets in a previous study. A reduction in food intake is known to reduce cancer incidence in a number of cancer models and is likely to have contributed to the decrease in expected cancer incidence in the current study. In mice with tumors, daidzein and equol diets significantly altered the serum levels of cytokines, IL-1β, IL-10, IFN-γ, and TNF-α compared to the control and soy germ groups suggesting that daidzein and equol may play a role in moderating tumoral inflammation. In conclusion, we determined that
pelleting the diets resulted in significantly decreased food intake and body weights and decreased cancer incidence. There was no difference in cancer incidence between diets, but we did see a significant effect of daidzein and equol on serum inflammatory cytokines in tumors which may suggest a reduction in inflammation in advanced PCa.

1 Additional tables and figures not included in this chapter are in Appendix B.
INTRODUCTION

Prostate cancer (PCa) is the most common cancer in men and is the second leading cause of cancer deaths in men in the U.S.\textsuperscript{2}. The incidence of PCa varies widely among regions in the world with the US, France, Barbados, and Sweden reporting some of the highest incidences and countries such as Japan, China, and South Korea reporting some of the lowest incidences\textsuperscript{3}. Risk factors for PCa include age, genetics, family history, race, smoking, obesity, and diet\textsuperscript{4,5}. Dietary factors have been widely hypothesized to account for differences in PCa rates between regions of the world. Immigration studies have shown a 3 to 5-fold increase in PCa risk in Asian-American immigrants compared to their native counterparts\textsuperscript{6}. These results suggest that PCa risk is relatively low in native Asian countries, but the risk is increased in Asian men living in Western countries\textsuperscript{6}.

Over the last 30 years, evidence that a diet rich in soy products may be protective against PCa, has been growing. Soy products contain an array of bioactive compounds including saponins, lignans, and the isoflavones genistein, daidzein, and glycitein. Intake of isoflavones varies geographically. Consumption of isoflavone-rich foods such as tofu, tempeh, and miso is common in Asian countries where dietary intakes of isoflavones have been estimated to range from 15 mg/day in China to 26-54 mg/day in Japan, while the average intake of isoflavones in Western countries is estimated at 3 mg/day\textsuperscript{7,8}.

Our lab recently demonstrated that consumption of a 2% soy germ diet reduced the incidence of PCa in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model by 34% compared to the control diets\textsuperscript{1}. Soy germ, the hypocotyl of the soybean, has a radically different isoflavone profile than whole soybeans. It contains considerably higher levels of daidzein and glycitein and relatively low levels of genistein when compared to whole soybeans.
Soy germ is currently utilized in dietary supplements, and food scientists have been investigating ways to incorporate it into functional foods for disease prevention\textsuperscript{11–14}. Therefore, it would be beneficial to determine the anticancer effects of soy germ and its mechanisms of action.

Genistein, the predominant isoflavone in soy products, has been investigated for its anti-proliferative, antioxidant, and chemopreventive properties in cell, animal, and human models\textsuperscript{9,10}. Although genistein has been associated with a protection against several cancers and other chronic diseases, there is a need to investigate the effects of other commonly-consumed isoflavones, daidzein, glycistein, and their metabolites.

Epidemiological studies suggest a reduced risk of PCa in men who consume soy products\textsuperscript{15}, and this effect appears to be especially pronounced in men who are “equol producers” (> 20 nM serum equol)\textsuperscript{16}. Daidzein can be metabolized by the gut microbiota into the weak estrogen-like compound, equol. It is not well understood if equol production is responsible for the protective effect observed with soy intake. Equol has been shown to have a higher binding affinity for estrogen receptor-β (ERβ) than daidzein\textsuperscript{17}. Equol has also been shown \textit{in vitro} and \textit{in vivo} to bind 5α-dihydroxytestosterone (DHT), thereby inhibiting its binding to the androgen receptor modulating androgen signaling in the prostate\textsuperscript{18}. This is significant because equol’s ability to be a selective estrogen modulator as well as an androgen antagonist makes it a promising target to study for hormonally-driven cancers like PCa.

In TRAMP mice, we previously demonstrated that prostatic equol was 39 times higher than genistein and 3 times higher than daidzein in mice consuming soy germ\textsuperscript{1}. The elevated concentration of equol in the prostate suggests that equol was a critical contributor to the anticarcinogenic effects of soy germ found in the TRAMP model\textsuperscript{1}. If the benefits from soy germ are primarily attributed to equol production, then an estimated 70-75% of the Western population
would not benefit from soy germ consumption as it is estimated that only 25-30% of the populations in Western countries are equol “producers” compared to up to 80% of individuals in China, Japan, and South Korea\(^\text{19}\).

Limited \textit{in vivo} studies have directly examined feeding equol and the incidence of PCa in rodents. Due to the previously observed protective effect of soy germ, we designed this study to follow-up on the results from Zuniga et al. and compare the effects of soy germ, daidzein, and equol in prostate carcinogenesis\(^1\). We hypothesized that equol was primarily responsible for the protective effect of soy germ, and that the soy germ, daidzein, and equol-fed mice would have reduced cancer incidence compared to the control group.

**MATERIALS AND METHODS**

**Animal Methods.** The University of Illinois Laboratory Animal Care Advisory Committee approved all animal procedures. Female and male heterozygous TRAMP (C57BL/6) mice from our colony were bred with FVB/NJ mice to obtain (TRAMPxFVB/NJ)\(F_1\) offspring for the study. A minimum of 28 mice per diet group were projected to be needed to reach statistical significance by power analysis (\(\alpha=0.05, \beta=0.8\)) for cancer incidence and two extra mice were added to each diet group to account for the occasional unforeseen death of study animals. Mice were genotyped via PCR-based DNA (Sigma-Aldrich, Saint Louis, MO) screening using established methods\(^1\). Offspring were weaned at 3 weeks of age, individually housed in shoebox cages under controlled conditions (12 h light/dark cycle, 22 °C, 60% humidity) and acclimated to a pelleted, modified semi-purified AIN-93G diet for one week. The modified AIN-93G included corn oil instead of standard soybean oil as the fat source. At 4 weeks of age, mice were randomized to consume one of four experimental diets: AIN-93G control, AIN-93G + 2% soy germ, AIN-93G + 82 ppm daidzein, or AIN-93G + 88 ppm equol. The daidzein diet included 92
ppm of purified daidzein to match levels found in a 2% soy germ diet. Equol was matched to daidzein equivalents found in a 2% soy germ diet with the assumption that 100% of the daidzein would be metabolized to equol by the highly efficient microbiota in the mouse\(^{20}\). Non-transgenic control littermates (n=10 per diet group) were included in the study to confirm the effects of the transgene.

Mice were weighed and individual food intake was measured when fresh food was provided weekly. At 18 weeks of age, mice were asphyxiated by CO\(_2\) and blood was collected via cardiac puncture. Serum was separated following centrifugation, aliquotted, frozen, and stored for analyses. The prostate was micro-dissected into individual lobes (anterior, ventral, and dorso-lateral). One half of each prostate lobe was fixed overnight in 10% phosphate-buffered formalin and then transferred to 70% ethanol for histological evaluation. All animals were thoroughly examined for gross metastases by trained research staff during the necropsy. A section of liver, lungs, and lymph nodes were fixed in 10% phosphate-buffered formalin and transferred to 70% ethanol after 24 hours for evaluation of micro-metastases. Liver, testes, spleen, gonadal adipose, and one half of each prostate lobe were flash frozen in liquid nitrogen and stored at -80 °C.

**Diet Formulation and Isoflavone Analysis.** Experimental diets were prepared as a custom AIN-93G formulation by Harlan Laboratories (Madison, WI). Diets were balanced for protein, carbohydrates, fat, energy, and fiber, provided 3.8 Kcal/g, and were stored at 4 °C in the dark. Experimental diet compositions are presented in Table 4.1. Soy germ was analyzed for daidzein isoflavone equivalents. Isoflavone content of the final experimental diets was analyzed by the National Center for Toxicological Research in Jefferson, AR using HPLC-UV. Briefly, 500 mg of diet was crushed and ground into a powder and placed in a 15 mL centrifuge tube. 2.5
mL of 80% methanol:water (80:20, v/v) was added to each tube. Samples were vortexed and then sonicated for 30 minutes. The supernatant was removed following centrifugation and placed into a 10 mL volumetric flask. The above methanol:water addition, vortex, sonication, and centrifugation was performed 3-5 more times and the supernatants were combined for each sample. The combined supernatant was filtered prior to HPLC analysis. Isoflavone analysis of prepared diets is presented in Table 4.2

**Serum Isoflavone Analysis.** Serum isoflavones were analyzed by the National Center for Toxicological Research in Jefferson, AR using HPLC-UV. The limits of detection of genistein, daidzein, and equol were 0.004, 0.002, and 0.03 µM respectively.

**Histopathology.** Formalin-fixed, paraffin-embedded prostate sections were stained with hematoxylin and eosin (H&E) and blindly evaluated by a pathologist using an established and published grading scheme. Each lobe of the prostate was evaluated for the most severe lesion and the most common lesion in each lobe. Additionally, sections of liver and lungs from mice with gross tumors and visible lymph node metastases were evaluated for the presence of micrometastases. Results were compared using Fisher’s exact test between treatment groups and the control.

**Immunohistochemistry.** Immunohistochemistry for proliferating cell nuclear antigen (PCNA) and cleaved caspase-3 (CC3) were performed on paraffin-embedded (4 µm) fixed sections of the dorso-lateral prostate lobe according to a previously published protocols. Briefly, slides were placed in a decloaking chamber and treated in a citrate buffer (pH 6.0) for 30 seconds at 125 ºC and 10 seconds at 90 ºC for antigen retrieval. In a BioGenex i6000 Automated Staining System (BioGenex, San Ramon, CA), endogenous peroxide was quenched with a 3% H₂O₂ solution for 15 minutes, slides were blocked with Power Block™ (BioGenex) for 10
minutes, avidin blocked for 15 minutes, and then incubated with rabbit ant-proliferating cell nuclear antigen (PCNA) antibody (Abcam, Cambridge, MA) or rabbit anti-cleaved caspase-3 (Cell Signaling, Danvers, MA) for 30 minutes and visualized using a SuperSensitive™ Link-Label IHC Detection System (BioGenex). Slides were stained with DAB and counterstained with hematoxylin. Mouse small intestinal tissue was used as the positive control for PCNA and mouse thymus tissue was used as the positive control for cleaved caspase-3. Negative controls were generated by omitting the primary antibody. Stained slides were scanned with a NanoZoomer 2.0-HT digital slide scanner (Hamamatsu, Bridgewater, NJ) with Olympus Uplansapo 20X objective at 40X digital zoom, resulting in 0.23 µm resolution. Images were captured with NDP view software (Hamamatsu). One image from the dorso-lateral lobe was blindly quantified and the proliferative index (PI) percentage (PCNA positive-stained nuclei/ total nuclei counted x 100) and apoptotic index (AI) percentage (number of activated caspase-3 cells/ total nuclei counted x 100) calculated. These indices were established by counting at least 1000 randomly selected cells from each image.

**Serum Testosterone.** Serum testosterone was measured in TRAMP mice fed control (n=10), soy germ (n=10), daidzein (n=10), and equol (n=10) diets. Serum testosterone was extracted from 150 µL of serum using di-ethyl ether. After the ether evaporated, a hexane and methanol extraction was used to extract the testosterone from other serum lipids for analyses. Serum testosterone was evaluated using Coat-A-Count total testosterone radioimmunoassay (Siemens TKTT2, Los Angeles, CA).

**Serum Cytokines.** Serum cytokines (IL-6, TNF-α, IL-1β, IL-10, IL-17A, and IFN-γ) were quantified using the Bio-Plex Pro Mouse Cytokine Th17 panel (Bio-Rad, Hercules, CA). 50 µl of serum was mixed with multi-colored magnetic beads contained in a single well.
Biotinylated detection antibodies were added to quantify analytes and samples were run on the Bioplex Cytometric Bead Analyzer in duplicates (Bio-Rad, Hercules, CA). Differences between groups were analyzed using 2-tailed ANOVA followed by post-hoc Dunnetts test and specific contrast statements.

**Global DNA Methylation.** DNA and RNA were simultaneously extracted from dorso-lateral prostates using the Qiagen All-Prep Mini kit (Qiagen, Valencia, CA). The MethylFlash Quantification Colorimetric kit (Epigentek, Farmingdale, NY) was used to quantify relative and absolute amounts of 5-methyl-cytosine according to the manufacturer’s protocol.

**RESULTS**

**Weight Gain and Feed Intake.** Body weight and food intake were measured weekly and recorded. All diets were readily consumed and food intake was not significantly different between groups (Table 4.3). Average food intake was similar to other studies of TRAMP mice consuming pelleted diets\(^{24,25}\). Body weights were not significantly different between groups. Organ weights of the urogenital tract, liver, lungs, testes, gonadal adipose, spleen, ventral prostate, anterior prostate, and dorso-lateral prostate were not different between groups (data not shown).

**Serum Isoflavones.** Genistein, daidzein, and equol were below the limit of detection in the AIN-93G control-fed mice. Genistein was only detected in mice consuming soy germ. Daidzein was only detected in the serum of mice fed soy germ or the daidzein diet and was significantly different between the two groups (p=0.048) (Table 4.4). Equol was detected in all the mouse serums from experimental diet groups except for the control diet and was not statistically different between groups. Differences in serum isoflavones between diets were analyzed using ANOVA and the Tukey-Kramer studentized range test (\(\alpha=0.05\)).
Histopathology. There were no differences in overall combined cancer incidence between diet groups (Figure 4.1). Mice fed the control, soy germ, daidzein, and equol diets had a 24%, 31%, 20%, and 28% incidence of cancer overall respectively. There was also no difference in cancer incidence by diet between anterior, ventral, or dorso-lateral lobes individually (data not shown). Furthermore, we were not able to detect any differences between pathology scores between diet groups (Table 4.5).

Immunohistochemistry. There was no statistical difference between AI (Figure 4.2) or PI (Figure 4.3) between diet groups in prostates with HGP. There was also no difference in AI between diets in tumors (Figure B.8, Appendix B).

Serum Testosterone. There were no statistical differences in serum testosterone levels between groups (Figure B.1 and B.2, Appendix B). Furthermore, we examined serum testosterone levels in wild-type non-transgenic mice (n=5/diet) to determine the impact of our diets on serum testosterone in a non-cancer model. There were no differences between groups, though the daidzein group was numerically lower than the other diet groups (Figure B.3 and B.4, Appendix B).

Serum Cytokines. Serum levels of IL-1β were significantly lower in mice with tumors fed daidzein and equol when compared to mice with tumors fed the control and soy germ diets (p<0.001) (Figure 4.4). Across all diets, there was no difference in IL-1β levels in high-grade PIN (HGP). Mice with tumors had significantly higher levels of serum IL-6 than mice with HGP (p=0.004), but there were no differences between dietary treatments (Figure 4.5). Mice with tumors fed soy germ had significantly increased levels of IL-10 compared to mice with tumors fed daidzein (p=0.04) or equol (p=0.02) (Figure 4.6). Mice with tumors fed the control diet were not statistically different than mice fed daidzein or equol diets. With TNF-α, mice with tumors...
fed soy germ and control had significantly higher TNF-α than mice fed daidzein (p=0.02) and equol (p=0.04) (Figure 4.7). Lastly, IFN-γ levels were significantly higher levels in mice with tumors fed daidzein (p=0.03) and equol (p=0.02) (Figure 4.8) compared to the control and soy germ diets. There were no differences in IL-17 levels between diets in either tumors or HGP (B.5, Appendix B).

Global DNA Methylation. Global DNA methylation was measured in TRAMP dorso-lateral prostates with confirmed HGP lesions (n=10/diet group). There was no difference in the percentage of 5-methyl cytosine relative to the reference standard between any of the four diet groups (Figure B.6, Appendix B). There was also no difference in the absolute quantification of 5-methyl cytosine between the four diets (Figure B.7, Appendix B).

DISCUSSION

Due to previous findings in our laboratory\(^1\), we hypothesized that diets incorporating 2% soy germ, daidzein, or equol would equally reduce the incidence of PCa in TRAMP mice compared to the control diet. We further hypothesized that the mechanism would be due to the isoflavone metabolite, equol. Contrary to our hypothesis, we did not detect any dietary differences in cancer incidence. To our surprise, mice in the control group had a 24% incidence of cancer whereas in a previous study in our lab, mice in the control group only had a 100% incidence of cancer at the same 18 week time point\(^1\). Despite using the same animal model, rodent colony, animal facility, sacrifice age, lot of soy germ, and animal procedures, we were not able to repeat the previous findings\(^1\). Similar levels of isoflavones were detected in the serum and diets from both studies. The only difference, aside from specific diets used, was the physical format that the diet was provided to the rodents. Zuniga et al. fed rodents a powdered version of
the AIN-93G-based diets, while the current study provided the similarly-formulated diets in pelleted form\(^1\).

The diet’s physical form resulted in drastically different food intakes between the studies. Zuniga et al. reported that mice consumed 5.7 grams/day while in the current study only 2.3 grams/day were consumed\(^1\). It should be noted that measurement of powdered food intake is less precise than with pelleted food intake, as powdered food tends to be distributed in bedding and in the cage, so at best, we can only get an estimate of powdered food intake in these studies. Pelleted food intake in the current study was consistent with other studies of TRAMP mice consuming pelleted diets\(^{24,25}\). The differences in food intake resulted in significantly different body weights between the two studies (Figure 4.9). Mice fed pelleted diets in the current study, were significantly lighter than mice fed powdered diets in the previous study as early as 8 weeks of age (p<0.001) and continuing until animals were sacrificed at 18 weeks of age (Figure 4.9) (p<0.001).

The associations between diet format and obesity has been examined extensively in rodent models\(^{26–28}\). Ford et al. described the association between food consumption and diet hardness in mice, and observed that increasing the hardness of food, negatively impacted growth and food intake\(^{29}\). Additionally, rats fed soft pellets developed obesity faster than their counterparts fed hard pellets\(^{30}\). Desmarchelier et al. examined the impact of feeding a control, high-fat, and Western diet to mice in either pelleted or powdered form\(^{26}\). The results of that study indicated that regardless of diet composition, all mice fed powdered diets developed similar weight gain\(^{26}\). This suggests that the texture of the food, rather than the nutrient composition, promoted hyperphagia and obesity\(^{26}\). Furthermore, in another study, long-term ingestion of powdered food induced hyperglycemia and systemic illness, increased adrenal gland activity,
and increased blood pressure in mice\textsuperscript{27}. This could be a result of easier access to powdered foods (bowls of food in the cage vs. food provided above the cage in a rack), ingestion of powdered food from fur while cleaning, and/or faster rates of absorption of powdered food\textsuperscript{27}. Together these findings suggest that the dietary format of food impacts the growth, development, and systemic health of healthy rodents. A diet in powdered format that contributes to excessive weight gain and systemic illness may promote carcinogenesis in a transgenic mouse model of PCa while pelleted diets might delay the onset of carcinogenesis.

It has been previously shown that TRAMP mice fed a high-fat Western diet have accelerated tumor growth and tumor burden compared to the control-fed mice\textsuperscript{31}. Alternatively, caloric restriction has been shown to reduce PCa incidence and progression in TRAMP mice. A 20\% dietary restriction beginning at 7 weeks of age resulted in significant reductions in PCa incidence at 11 and 20 weeks of age in TRAMP mice\textsuperscript{32}. Additionally, TRAMP mice who were intermittently or chronically calorically restricted had a decreased incidence of PCa\textsuperscript{24}. While in the current study, the mice were not calorically restricted and were allowed access to their food \textit{ad libitum}, they consumed nearly 50\% fewer grams of food/day than mice fed powdered food and weighed significantly less than mice fed powdered food\textsuperscript{1}. This reduction in food intake might account for the reduction in cancer incidence observed.

However, changes in caloric intake and energy balance and expenditure also alters cancer incidence in TRAMP mice. While caloric restriction alone has been shown to reduce cancer incidence in the TRAMP model, changes in energy balance and excess caloric retention contribute to cancer progression. Mice housed at 22 °C expend more energy and have lower body mass than mice housed closer to their thermoneutral zone (30-35 °C)\textsuperscript{33}. Interestingly, when mice are housed a 22 °C, they consume 30\% more calories, but have less body mass than mice housed
at 27 °C[33]. This suggests that mice consume more food to compensate for thermoregulatory demands, but that energy balance plays a role beyond food intake. The use of pelleted diets in the current study and the temperature mice were housed at (22 °C) combined to produce an environment where mice had to expend energy to consume food and expend energy to maintain thermoregulation, ultimately keeping their body weight lower. These results suggest that pelleted diets fed ad libitum resulted in decreased body weight and decreased cancer incidence compared to mice fed powdered diets ad libitum\(^1\). The decrease in body weight was likely a contributing factor to the decrease in expected cancer incidence in all dietary treatment groups.

Evidence exists that inflammation plays a critical role in the development and progression of PCa\(^{34}\). We measured serum levels of pro-inflammatory and anti-inflammatory cytokines in TRAMP mice displaying gross primary prostate tumors and compared them to mice with histopathologically confirmed high-grade PIN (HGP) prostates across all four diets. While cancer incidences were not different between our diets, serum levels of cytokines varied significantly by diet when we compared preneoplastic prostates to mice with developed prostate tumors. Interestingly, we observed a pattern in several serum cytokines measured that the daidzein and equol diets had significantly different serum cytokine levels than the soy germ and control diets. In mice with tumors, the daidzein and equol-fed groups had significantly lower levels of circulating IL-1β than the control and soy germ groups (Figure 4.2). IL-1β has been shown in C4-2 PCa cells to drive PCa progression by inhibiting AR expression and inducing p62\(^{35}\). Additionally, tumor IL-1β levels remained independent prognostic factors in human prostatectomy samples, suggesting that IL-1β might be indicative of clinical outcomes\(^{36}\). The significant decrease in IL-1β levels in mice with tumors by daidzein and equol diets may suggest that these diets may have anti-inflammatory properties beneficial in delaying or reducing PCa
progression to advanced or castration-resistant PCa. In contrast, IL-1β has also been shown in LNCaP prostate cancer cells to inhibit proliferation and reduce PSA production through NF-κB activation\(^\text{37}\). Consistent with our histopathological data and proliferation results, there was no difference in IL-1β serum levels between diets in mice with prenoplasic HGP prostates. Therefore, it appears that IL-1β may have dual action in PCa in that it confers protection in precancerous stages of PCa and may contribute to the progression of advanced PCa.

Additionally, IFN-γ and TNF-α were also significantly reduced by the daidzein and equol diets in serum of mice with tumors than in the control and soy germ groups (Figure 4.8, Figure 4.7). TNF-α is a major pro-inflammatory cytokine secreted in the tumor micro-environment that can activate PCa progression signaling pathways\(^\text{36}\). Prostatic expression of TNF-α from human prostatectomy samples has also been suggested to indicate clinical outcomes in PCa\(^\text{36}\). The impact of consumption of soy foods on TNF-α levels have yielded mixed results in the literature\(^\text{38,39}\).

IFN-γ is a type II interferon produced by CD4+ lymphocytes and natural killer (NK) cells\(^\text{40}\). In a mouse model of PCa, IFN-γ treatment led to a 3-fold increase in apoptosis in the primary prostate tumor\(^\text{40}\). Moreover, IFN-γ has been shown to promote anti-tumor responses in several cancers\(^\text{41}\). In this study, we observed a significant decrease in IFN-γ in serum from mice with tumors fed daidzein and equol (Figure 4.8). While there has been no correlation between levels of IFN-γ and cancer severity, the decreased IFN-γ levels in the daidzein and equol-fed groups warrant further investigation as increased IFN-γ levels are associated with protection against PCa.

Daidzein and equol also significantly reduced serum levels of IL-10 in mice with tumors compared to the soy germ diet (Figure 4.6). IL-10 is generally known to be an anti-
inflammatory cytokine, but studies in human prostate tissue have shown it to be increased in advanced PCa\(^42\). This further indicates that daidzein and equol may potentially mediate the tumoral inflammatory environment.

Serum levels of IL-6 were significantly increased across all diets in mice with tumors than in mice with HGP (Figure 4.5). IL-6 has been shown to activate AR and enhance PCa growth\(^43\). While there was no effect of diet on serum IL-6, the increase in IL-6 in mice with prostate tumors is consistent with the literature. An IL-6 super-antagonist has been shown to inhibit the growth of PC-3 xenografted cells in mice\(^43\). This suggests that therapies targeted to decrease levels of IL-6 may be beneficial in the future against PCa.

Interestingly, we did not observe any reductions in serum levels of cytokines in mice fed the soy germ diet, but we did observe differences by the daidzein (matched for daidzein equivalents in the 2% soy germ diet) and equol diets. While the primary isoflavone in soy germ was daidzein, the interactions between daidzein, glycitein, and genistein from the whole soy germ along with saponins, lignans, and vitamin E in the soy germ food matrix may have impacted soy germ’s efficacy on modulating serum cytokines. Together, the main outcome of cancer incidence was not different between diets; however, daidzein and equol diets reduced levels of pro-inflammatory cytokines in the serum of mice with tumors suggesting that these two diets may be beneficial in reducing systemic inflammation in advanced PCa.

In this study, cancer incidence was remarkably lower in the TRAMP model than previous studies in our lab\(^1,44\). The reduction in cancer incidence may be explained by a change in dietary format from powdered to pelleted diets. The combination of feeding pelleted diets and housing mice individually below their thermonuetral zone may have shifted energy balance in TRAMP mice to only eat enough for maintenance and growth. Future studies utilizing pelleted diets might
detect differences between dietary treatments by prolonging the sacrifice age further than 18 weeks to allow for cancer development or switching back to powdered diets. Despite null results regarding cancer incidence, we observed a significant effect of daidzein and equol on serum cytokines in mice with prostate tumors. This may suggest that daidzein and equol diets, but not a whole soy germ diet, may have anti-inflammatory properties in advanced stage PCa. Future studies in TRAMP mice should examine serum cytokine levels as well as prostatic and tumoral mRNA expression of cytokines to confirm these findings.

ACKNOWLEDGMENTS

The authors would like to thank Frutarom for donation of the soy germ powder and Dr. William Helferich for the donation of the purified daidzein and equol. We would also like to thank Dr. Smille and Dr. Wang at the University of Mississippi for analyzing the soy germ, daidzein, and equol and Dr. Doerge at the FDA’s National Center for Toxicological Research for analyzing serum and diet isoflavones. This work was supported by National Institutes of Health P50 AT006265.
### Table 4.1. Composition of Experimental Diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control</th>
<th>2% Soy Germ</th>
<th>Daidzein</th>
<th>Equol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>39.7</td>
<td>39.8</td>
<td>39.7</td>
<td>39.7</td>
</tr>
<tr>
<td>Casein</td>
<td>20</td>
<td>19</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>13.2</td>
<td>13.2</td>
<td>13.2</td>
<td>13.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Fiber</td>
<td>5</td>
<td>4.3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Choline Bitrate</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>7</td>
<td>6.5</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>TBHQ, antioxidant</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.0014</td>
</tr>
<tr>
<td>Soy Germ</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0</td>
<td>0</td>
<td>0.0092</td>
<td>0</td>
</tr>
<tr>
<td>Equol</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0088</td>
</tr>
</tbody>
</table>

*a 2% Soy Germ diet contains 71 ppm daidzein equivalents, 68 ppm glycitein equivalents, and 35 ppm genistein equivalents

*b Daidzein diet contains 92 ppm daidzein equivalents

*c Equol diet contains 88 ppm equol equivalents

*d Non-nutritive cellulose

*e AIN-93G-MX formation

*f AIN-93G-VX formation

*g Frutarom SoyLife® Complex Micro
Table 4.2. Isoflavone Analyses of the Prepared Diet (ppm)

<table>
<thead>
<tr>
<th>Diet</th>
<th>AIN-93G + 2% Soy Germ (ppm)</th>
<th>AIN-93G + 92 ppm Daidzein (ppm)</th>
<th>AIN-93G + 88 ppm Equol (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>35</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Daidzein</td>
<td>71</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>Glycitein</td>
<td>68</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Equol</td>
<td>0</td>
<td>0</td>
<td>80</td>
</tr>
</tbody>
</table>

Values are the mean of n=5.

Table 4.3. TRAMP Body Weights and Food Intake

<table>
<thead>
<tr>
<th></th>
<th>AIN-93G Control</th>
<th>AIN-93G + 2% Soy Germ</th>
<th>AIN-93G + 92 ppm Daidzein</th>
<th>AIN-93G + 88 ppm Equol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beginning Body Weight (g)</td>
<td>20.2 ± 3.7</td>
<td>19.4 ± 3.6</td>
<td>20.3 ± 3.8</td>
<td>20.6 ± 3.8</td>
</tr>
<tr>
<td>Ending Body Weight (g)</td>
<td>27.3 ± 5</td>
<td>27 ± 5</td>
<td>27.7 ± 5</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>Average Food Intake (g/day)</td>
<td>2.3 ± 0.4</td>
<td>2.3 ± 0.4</td>
<td>2.3 ± 0.4</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>n</td>
<td>30</td>
<td>29</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM.

Table 4.4. TRAMP Serum Isoflavone Analyses

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>Serum (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diet</td>
</tr>
<tr>
<td>Genistein</td>
<td>ND</td>
</tr>
<tr>
<td>Daidzein</td>
<td>ND</td>
</tr>
<tr>
<td>Equol</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM

ND = Not detected in the serum

Superscript letters in the same row indicate a significant difference between groups (p=0.048).

n=7 per group
**Figure 4.1.** Overall Prostate Cancer Incidence in TRAMP mice

Different colored bars represent the distribution of cancer severity among diets.

n=29-30/diet group

### Table 4.5. TRAMP Histopathology Results as a Percentage of Total Prostatic Lesions by Diet

<table>
<thead>
<tr>
<th>TRAMP</th>
<th>PIN</th>
<th>Adenocarcinoma</th>
<th>Prostate Cancer (WD-PD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>NP</td>
<td>LGP</td>
</tr>
<tr>
<td>AIN-93G Control</td>
<td>29</td>
<td>7%</td>
<td>0%</td>
</tr>
<tr>
<td>AIN-93G + 2% Soy Germ</td>
<td>29</td>
<td>0%</td>
<td>3%</td>
</tr>
<tr>
<td>AIN-93G + 82 ppm Daidzein</td>
<td>29</td>
<td>3%</td>
<td>7%</td>
</tr>
<tr>
<td>AIN-93G + 88 ppm Equol</td>
<td>29</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

NP = Normal Prostate, LG = low grade PIN, MG = moderate grade PIN, HG = high-grade PIN, PLL = phyllode-like lesions, WD = well differentiated, MD = moderately differentiated, PD = poorly differentiated.

Results are the incidence of each stage of pathology and overall incidence (sum of WD-PD) within dietary groups.
Figure 4.2. TRAMP Prostatic Apoptotic Index

![Prostatic Apoptotic Index](image)

Values are means ± SEM.

n=5/diet groups

Figure 4.3. TRAMP Proliferation Index

![Prostatic Proliferation Index](image)

Values are means ± SEM.

n=5/diet groups
Values are means ± SEM.

n=6-7/diet groups

Different capital letters indicate significant differences between diet groups in tumors (p<0.05).
**Figure 4.5.** TRAMP Serum IL-6

![Graph showing Serum IL-6 levels across different diets and tumor stages.](image)

Values are means ± SEM.

n=6-7/diet groups

Different capital letters indicate significant differences between tumor and HGP (p<0.05).

**Figure 4.6.** TRAMP Serum IL-10

![Graph showing Serum IL-10 levels across different diets and tumor stages.](image)

Values are means ± SEM.

n=6-7/diet group

Different capital letters indicate significant differences between diet groups within tumors (p<0.05).
Figure 4.7. TRAMP Serum TNF-α

Values are means ± SEM.

n=6-7/diet groups

Different capital letters indicate significant differences between diet groups within tumors (p<0.05).
Figure 4.8. TRAMP Serum IFN-γ

Values are means ± SEM.

n=6-7/diet groups

Different capital letters indicate significant differences between diet groups within tumors (p<0.05).
Figure 4.9. TRAMP Body Weight Comparison between Zuniga et al. and Conlon et al.

Values represent the mean body weight across all diets at each week of age.

* Represents a significant difference (α<0.05) between Zuniga et al. and the current study, Conlon et al. at specific time points.

Both studies n=29-31 weighed at each time point.
REFERENCES


CHAPTER 5

Summary and Future Directions

PCa is the most commonly diagnosed male cancer in the United States. There is a growing body of evidence that suggests a diet rich in fruits and vegetables, specifically tomato and soy, may protect against PCa. While many studies have demonstrated the effectiveness of the individual components of tomatoes and soy, carotenoids and isoflavones, in cancer development and progression, people primarily consume these bioactives from whole foods. Therefore, not only should researchers study the effects of dietary bioactives, they should also consider the matrix of the whole food and the co-consumption of other nutrients and food components within a meal.

In Chapter 2, we aimed to determine if the saturation and chain length of dietary fat co-consumed with a 10% TP diet would impact the bioavailability of tomato carotenoids and affect their tissue distribution in the Mongolian Gerbil. In this study, we observed differential tissue accumulation patterns of dietary carotenoids. The specific carotenoid profiles in each tissue may reflect differences in metabolism of carotenoids in tissues. Furthermore, we observed that the coconut oil diet increased carotenoid bioaccumulation in all tissues measured except for the spleen and skin compared to safflower oil. This increased bioaccumulation of tomato carotenoids may have been the result of increased carotenoid solubility in the intestinal lumen, enhanced portal absorption with medium-chain triglycerides from coconut oil, and/or a reduction in hepatic cholesterol uptake induced by coconut oil. Nonetheless, this finding is significant because it emphasizes that the type of dietary fat co-consumed with carotenoid-containing foods is important in the tissue distribution of carotenoids. While coconut oil consumption resulted in the greatest tissue accumulation of tomato carotenoids, it also significantly increased serum
cholesterol in this study. Therefore, a decision to incorporate coconut oil into diets to increase carotenoid bioaccumulation should be balanced with the negative consequences of saturated fat on overall health. Future studies should focus on identifying the mechanisms for differential carotenoid accumulation between tissues and investigate whether shifts in tissue fatty acid profiles enhanced carotenoid accumulation.

In Chapter 3, we evaluated if a 10% TP intervention could reduce PCa incidence in the TRAMP model. Previous evidence from epidemiological and animal studies suggested that a diet rich in tomato products may protect against PCa. However, previous animal studies model lifelong dietary consumption of TP or lycopene. Because the use of complementary and alternative medicine is prevalent among cancer patients and may not be initiated until cancer diagnosis, we evaluated if a 10% TP diet could decrease the risk for PCa when fed post-puberty when preneoplastic lesions were present. All TRAMP mice were fed the control diet (AIN-93G) from 3 weeks of age to 8 weeks of age when they were randomized to either continue consuming the control diet or consume the experimental diet (AIN-93G + 10% TP) until 12, 16, or 20 weeks of age. We found no change in overall cancer incidence by 10% TP diet at 12, 16, or 20 weeks of age. We did, however, observe a significant increase in HGP and a significant decrease in PD lesions by TP diet only at 16 weeks of age, although total cancer incidence at 16 weeks of age was not significantly different. This modest change may suggest that a 10% TP diet intervention delayed the progression of precancerous lesions to cancerous lesions at 16 weeks of age.

The lack of a dramatic effect in cancer incidence by TP may have been the result of two variables in our study: 1) the tomato carotenoid profile of the diet and 2) the timing of our intervention. The carotenoid content of the TP fed contained 30-fold less lycopene than our previous TRAMP study which might suggest there is a threshold of lycopene necessary to reduce
carcinogenesis in this model\textsuperscript{1,2}. However, tissue lycopene concentrations at 16 and 20 weeks of age were similar to a previous study using TRAMP mice in our lab, suggesting that tissue accumulation of carotenoids may reach a saturation point in tissues, including the prostate.

The second variable in our study was the timing of our intervention. The timing of our intervention may have been too late in carcinogenesis in TRAMP mice. Prior research by our group has shown that the TRAMP genotype altered several prostatic genes involved in cell-cycle and cell growth by 10 weeks of age\textsuperscript{3}. Furthermore, 6 weeks of a 10\% TP diet altered expression of genes involved in androgen signaling and synthesis in the prostate of TRAMP mice by 10 weeks of age\textsuperscript{3}. The dietary intervention in the current study was initiated at 8 weeks of age when mice had confirmed low- and moderate-grade PIN lesions in the prostate (confirmed by sacrificing a small sub-cohort of mice at 8 weeks of age) and after the onset of puberty. Carcinogenesis in the TRAMP model begins in response to androgens at puberty, or around 6 weeks of age. The response of the rat probasin promoter to androgens at puberty results in the expression of the Simian virus 40 large T antigen in the prostate epithelium. This results in the suppression of tumor suppressors, p53 and Rb, and uncontrolled proliferation and mutations in the prostate. Perhaps beginning a TP diet intervention after the onset of puberty during a time when PIN lesions were developing was too late to substantially impact carcinogenesis in this model. Equally plausible, is that the combination of a low-lycopene-containing TP and a late intervention time contributed to our results. While it appears that lycopene reaches saturation in some tissues with long-term feeding, TP diets may only be effective in this model when they contain enough lycopene to modulate androgen signaling and metabolism early in carcinogenesis, a threshold still to be determined. As reviewed Chapter 3, animal studies providing 28 ppm lycopene\textsuperscript{4} and 12.3 ppm lycopene (the current study), were ineffective in
reducing the risk of PCa in TRAMP mice. However when TP contained more lycopene (132-286 ppm), they were effective at reducing PCa incidence\textsuperscript{1,5}. Nonetheless, this study supports the importance of lifelong consumption of tomato products for PCa prevention. Future studies should aim to identify if there is a critical window of lycopene exposure necessary to alter PCa in this model and if this impacts its translation to human PCa outcomes.

In Chapter 4, we aimed to further elucidate the mechanism by which a 2\% soy germ diet reduced the incidence of PCa in the TRAMP model\textsuperscript{1}. We hypothesized that the effect of soy germ was primarily due to the microbial metabolite of daidzein, equol. TRAMP mice were randomized at weaning to consume one of four pelleted study diets: AIN-93G, AIN-93G + 2\% soy germ, AIN-93G + 92 ppm daidzein, or AIN-93G + 88 ppm equol until 18 weeks of age. Diets were pelleted to better assess food intake. To our surprise, only a fraction of the mice in this study had cancerous lesions, and the majority of mice had pre-neoplastic lesions at sacrifice. This effect was surprising as we designed this study to further investigate the results of a previous study in our lab\textsuperscript{1}. The only difference between studies, aside from treatment groups, was the physical format we provided the study diets to the mice.

As a result, providing study diets in pelleted form significantly reduced food intake and impacted the outcomes of the study. Other labs have shown that mice who consumed powdered diets gained more body weight, had increased blood pressure, and displayed other systemic effects than mice fed pelleted diets\textsuperscript{6,7}. When we compared the current study’s body weight values with mice from the Zuniga et al. study, our mice had significantly reduced body weights. We conclude that mice in the current study failed to develop cancer to the extent expected as mice on the same experimental protocol (aside from dietary treatment groups and diet form), because they gained less body weight as a result of altered energy expenditure and a reduction in food
intake. Future studies should take dietary format into consideration during the design of the study and if pelleted diets are to be utilized, the sacrifice age should be adjusted to ensure expected cancer incidence rates in control mice.

Additionally, we investigated the effects of soy germ, daidzein, and equol on serum cytokines in a sub-set of TRAMP mice with HGP and tumors. Interestingly, we observed that daidzein and equol-fed mice with tumors had significant reductions in IL-1β, IL-10, and TNF-α. These effects suggest that daidzein and equol diets reduced systemic inflammation in mice with advanced PCa. In contrast, daidzein and equol diets also significantly reduced serum levels of IFN-γ. Increased levels of IFN-γ has been thought to be protective in PCa prevention, so the reduction of this cytokine by the daidzein and equol diets was surprising\(^8,9\). While this study only investigated serum levels of cytokines, future studies should aim to determine if prostatic or tumoral mRNA expression of these cytokines are affected by diet.

In conclusion, people consume foods as parts of meals. While it’s important to determine mechanisms of specific bioactives from whole foods, there is a need to consider the effects of factors during a meal which may limit or enhance the bioavailability of bioactives. Much remains to be known about how diet can impact PCa development and progression over the lifespan and its possible mechanisms for protection. But the long latency period between PCa diagnosis and PCa treatment provides an ideal window for dietary modifications.
REFERENCES


APPENDIX A

Supplemental Tables and Figures

Figure A.1. TRAMP Serum Testosterone by Age and Diet

Values represent the mean ± SEM

n=20 per diet per age

# Indicates a significant difference (p<0.05) from the control diet within each age.

Figure A.2. TRAMP Prostatic mRNA Expression of Androgen Receptor by Age and Diet

Values represent the mean ± SEM.

# Indicates a significant difference between ages (p < 0.05).
Figure A.3. TRAMP Prostatic Androgen Receptor Expression by Pathology

Prostatic mRNA Expression of Androgen Receptor by Pathology and Diet

Values represent the mean ± SEM.

# Indicates a significant difference between pathologies (p < 0.05).

Figure A.4. TRAMP Prostatic mRNA Expression of 5α-Reductase I and II by Diet and Pathology

Prostatic mRNA Expression by Pathology

Values represent the mean ± SEM.

# Indicates a significant difference in 5α-Reductase II expression between pathologies (p < 0.05).
Figure A.5. TRAMP Prostatic mRNA CYP17A1 Expression by Diet and Pathology

Values represent the mean ± SEM.

# Indicates a significant difference between pathologies (p < 0.05).

Figure A.6. TRAMP Prostatic mRNA Expression of HSD17β2 and HSD17β3 by Diet and Pathology

Values represent the mean ± SEM.

# Indicates a significant difference between pathologies in HSD17β3 levels (p < 0.05).
**Figure A.7.** TRAMP Prostatic mRNA Expression of msmb by Diet and Pathology

Values represent the mean ± SEM.

# Indicates a significant difference between pathologies (p < 0.05).
**Figure A.8.** 3-D Prostate Tumor Volume vs. Gross Tumor Mass

Data points represent individual animal values

n=10

**Figure A.9.** Longitudinal 3-D Volumes of Mouse Prostate Tumors

Values represent the mean ± SEM

Tomato (n=5), Control (n=6)
Figure A.10. Gross Prostate Tumor Volume vs. 3-D Tumor Volume

Data points represent individual animal values

n=7

Figure A.11. Longitudinal 3-D Volumes of Mouse Prostates without Tumors

Values represent the mean ± SEM

Tomato (n=9), Control (n=8)
APPENDIX B

Supplemental Tables and Figures

**Figure B.1. TRAMP Serum Testosterone**

![TRAMP Serum Testosterone](image1)

Values represent mean ± SEM.

n=10/diet group

**Figure B.2. TRAMP Serum Testosterone**

![TRAMP Serum Testosterone](image2)

Values are individual data points.

**Figure B.3. Wild-type Serum Testosterone**

![Wild Type Serum Testosterone](image3)

Values represent mean ± SEM.

n=10/diet group

**Figure B.4. Wild-type Serum Testosterone**

![Wild Type Serum Testosterone](image4)

Values are individual data points.
Figure B.5. TRAMP Serum IL-17

Serum IL-17

Values are means ± SEM.

n=6-7/ diet group

Different letters indicate significant differences between diet groups with the same pathology (p < 0.05).
**Figure B.6.** Relative Prostatic Global DNA Methylation

Values represent mean ± SEM

n=10/diet group

**Figure B.7.** Absolute Prostatic Global DNA Methylation

Values represent mean ± SEM

n=10/diet group
Figure B.8. Tumoral Apoptotic Index

Values represent mean ± SEM

n=5/diet group