CHEMOPREVENTIVE EFFECTS OF CURCUMIN AND GREEN TEA ON B[A]P-INDUCED CARCINOGENESIS IN THE HAMSTER CHEEK POUCH

A Thesis

by

JIMI LYNN BRANDON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2005

Major Subject: Veterinary Microbiology

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ABSTRACT

Chemopreventive Effects of Curcumin and Green Tea on

B[a]P-induced Carcinogenesis in the Hamster Cheek Pouch. (May 2005)

Jimi Lynn Brandon, B.S., Texas State University

Co-Chairs of Advisory Committee: Dr. George Stoica
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The present study was carried out to examine the chemopreventive effects of curcumin and green tea polyphenols on the hamster cheek pouch carcinogenesis model. This model of oral carcinogenesis has been widely used in chemoprevention studies, however, these studies have been limited to the use of DMBA as the carcinogenic agent. We have developed a protocol of carcinogenesis in the hamster cheek pouch using B[a]P, a broadly distributed environmental carcinogen, formed as a by-product of the combustion of organic materials including cigarette smoke. B[a]P- induced tumors in the hamster cheek pouch are primarily endophytic squamous cell carcinomas that closely resemble squamous cell carcinomas of the human oral mucosa. The cheek pouch of male Syrian hamsters were treated topically for eight weeks with 0.6% curcumin, 6.0% curcumin, 2.5% green tea polyphenols, or 5.0% green tea polyphenols, 3 times per week 30 minutes prior to the application of 2.0% B[a]P.

The animals were sacrificed 24 hours and 72 hours after the last treatments. Short-term mechanistic markers of malignant progression were used to determine effects of each compound. Cellular proliferation, assessed by bromodeoxyuridine (Brdu) incorporation, p53 protein accumulation, and apoptotic activity were evaluated. The results of the present study demonstrated that 0.6% curcumin and 2.5% green tea

polyphenols had strong inhibitory effects on cellular proliferation and p53 protein accumulation. And 6.0% curcumin and 5.0% green tea polyphenols appeared to induce apoptosis. Our data suggest that curcumin and green tea polyphenols may have a plausible chemopreventive effect on oral carcinogenesis in the hamster cheek pouch model.

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INTRODUCTION

Oral Squamous Cell Carcinoma

Oral cancer is the sixth most common cancer in the world [1], and continues to be a growing problem. Oral cancer is considered to be a subdivision of head and neck cancer, and includes cancers of the lips, tongue, salivary glands, gum, mouth, pharynx, oropharynx, and hypopharynx. In the year 2002, there were almost 30,000 new cases of oral cancer reported in the United States, and 7,400 oral cancer related deaths [2] (http://seer.cancer.gov/csr/1975-2001/) with similar estimates for 2004. One of the most astounding statistics is a 56% 5-year survival rate in oral cancer patients[2]. Over the last several decades the incidence of many cancers has been declining and their prognoses have been improving. Unfortunately, oral cancer has remained steady during this period, and has recently begun to increase in the young male population. Incidence rates for oral cancer are two to four times higher among men than women [2]. In the past, oral cancer has primarily affected middle and older-aged individuals but the effected ages are decreasing, primarily because of the increased use of smokeless tobacco among young men. Approximately 11% of high school male students in the United States use smokeless tobacco [3], and 5.6% of middle school male students in the United States currently use smokeless tobacco [4]. According to the United States Department of Agriculture, the output of snuff, a smokeless tobacco put between the labia oris (bottom)

This thesis follows the style of *Carcinogenesis*.

lip) and the mandibular gingiva or the buccal mucosa, has risen over 26% in the past 10 years, from 59.1 million pounds in 1993 to 74.7 million pounds in 2003 [5]. These increases have recently led us to the importance of studying and developing new chemoprevention approaches in oral cancer. There are many factors that may contribute to this disease, but of these the most popular are the use of tobacco products, excessive alcohol consumption, and a poor, unbalanced diet. All forms of tobacco use, including smoking and smokeless tobaccos, have been linked to oral squamous cell carcinoma. Of all etiologic factors of oral cancer tobacco is regarded as the most important [6]. Excessive alcohol consumption is also problematic. Alcohol is not considered a carcinogen but when used excessively it can permeablize cellular membranes allowing carcinogens from tobacco and smoke to enter the cell resulting in damage to cellular structures as well as to the DNA [7-10]. It may also activate the enzymatic machinery that, in turn, activates carcinogens found in the environment [7].

Approximately 90% of oral cancers are squamous cell carcinomas. The development of squamous cell carcinomas is a multiple step process. As in all cancers oncogenes must be activated and tumor suppressor genes must be inactivated. These genetic alterations are often due to chronic exposure to carcinogens, promoters, and irritants. The lack of regulatory mechanisms due to the genetic alterations of oncogenes and tumor suppressor genes leads to dysfunction of the homeostatic mechanisms such as increased proliferation and/or decreased apoptosis and differentiation. As genetically altered cells proliferate they form premalignant foci in which the altered cells are confined by the basement membrane of the dermis. Eventually the foci can become

larger and acquire the ability to break through the basement membrane, invade adjacent tissues, and even metastasize to different parts of the body.

One of the earliest signs of oral carcinogenesis is leukoplakia, a white precancerous lesion found in the oral cavity that can often progress into squamous cell carcinoma. Leukoplakia is associated with continuous use of smokeless tobacco. Clinically it is a white lesion in the oral mucosa. Histopathologically, the lesion has hyperkeratosis, hyperplasia and stromal inflammation. More chronic lesions often have dysplastic changes [6]. Erythroplakia, a red precancerous lesion, is reported to have an even higher risk of progression [11]. Histologically, erythroplakia are similar to leukoplakia but are more dysplastic and vascularized. Most precancerous lesions are reversible if exposure to the tobacco products is ceased and lesions are treated early, however, some of these lesions will continue to progress into squamous cell carcinomas. Because it is unknown which of these lesions will transform it is of great importance to research and identify early biomarkers of progression to be used to determine cellular growth rates, death rates, and genetic damage or alterations. These biomarkers could further assist in determining prognosis and treatment.

Oral cancers can be categorized by way of their effected tissues. Carcinomas of the lips account for 25-30% of all oral cancers [6]. Carcinomas of the lower lip are more common than those of the upper lip. In addition, they seem to grow slower and have a better prognosis than lesions of the upper lip [6]. Poorly differentiated lesions can metastasize to local lymph nodes [6]. Squamous cell carcinoma of the tongue is the most common oral malignancy accounting for 25-40% of all intraoral (excluding lips)

malignancies [6]. These lesions are more asymptomatic and when finally diagnosed have usually metastasized to lymph nodes in the neck, and occasionally to the lung or the liver. Lesions can also develop on the floor of the mouth. These squamous cell carcinomas account for 15-20% of intraoral lesions. Metastasis often occurs to the submandibular lymph nodes [6]. Approximately 10% of oral squamous cell carcinomas develop in the buccal mucosa and gingiva. The lesions are usually slow growing, well differentiated and rarely metastasize resulting in a good prognosis [6]. Squamous cell carcinoma of the soft palate accounts for 10-20% of intraoral lesions. Metastasis often occurs to the cervical lymph nodes leading to an extremely poor survival rate, dropping from 50% to 20% [6]. Most oral squamous cell carcinomas are well differentiated producing keratin pearls, and invasion may occur into the subjacent structures. Some variations between tumors include the number of mitosis, nuclear pleomorphism, and keratinization. Poorly differentiated lesions often have lesser amounts of keratin and are of greater health concerns [6].

Grading of tumors is determined by differentiation of the tumor cells. Well differentiated lesions are often less aggressive grading a lower score while poorly differentiated lesions are more aggressive. All diagnosed oral cancers must be staged to determine treatment and prognosis. Staging of cancer is done according to the International Union Against Cancer (UICC) classification system for oral cancer [12]. Each parameter is evaluated and categorized. Using these parameters tumors are staged accordingly:

*Tumor
T1=Tumor less than 2 centimeters in diameter
T2=Tumor 2-4 centimeters in diameter
T3=Tumor greater than 4 centimeters in diameter
T4=Tumor invades adjacent structures
*Nodes
N0=No palpable nodes
N1=Ipsilateral palpable nodes
N2=Contralateral or bilateral nodes
N3=Fixed palpable nodes
*Metastasis
M0=No distant metastasis
M1=Clinical or radiographic evidence of metastasis

Stage I - T1 N0 M0
Stage II - T2 N0 M0
Stage III - T3 N0, T1-3 N1, and M0
Stage IV - T4 and N, T1-3 N2-3, any T any N M1

The first strategy to reducing this cancer is for all humans to stop excessive use of tobacco and alcohol, however, that is not always the most realistic solution, therefore the development of new strategies to prevent these cancers from emerging is of the utmost importance now and in the future.

Hamster Cheek Pouch

In 1919, Chinese hamsters were introduced into medical research. However, by 1930, the hamsters became unsatisfactory because they would not breed in captivity. Researchers decided to go to Syria to capture native hamsters to take back to their labs. One mother and ten young were captured. Nine of the young made it back to the University. Five of these eventually gnawed their way out of their cages leaving the researchers with only four offspring. These four animals proved to be very prolific, and soon Syrian (Golden) hamsters (Cricetus auratus) were being shipped to other countries. This breed of hamster was soon designated as "the" laboratory hamster. It is a convenient size, it is very adaptable to laboratory conditions and it has a gentle disposition [13].

The pouch of the Syrian hamster is located subcutaneous on each lateral side of the head and neck. The pouches are muscular sacs that open from the mouth and extend toward the shoulder. They measure approximately 35-40 millimeters in length and are 4-8 millimeters wide when empty. When full of food they can measure up to 20 millimeters wide [13]. These pouches have been used for decades to study the development of oral squamous cell carcinomas. In 1954, Salley began producing squamous cell carcinomas in the cheek pouch [14]. In 1961, Morris standardized conditions to produce oral squamous cell carcinomas in the hamster cheek pouch using 7,12-dimethylbenz[a]anthracene (DMBA) as the carcinogen [15]. In years to follow, other researchers have continued studying chemically induced carcinogenesis in this model and have conducted numerous chemoprevention studies [16-20].

Benzo[a]Pyrene

Benzo[a]pyrene (B[a]P), a member of the polycyclic aromatic hydrocarbon (PAH) family, is a Class 2A carcinogen and is widely distributed throughout the world's environment. According to the IARC Carcinogen Classification a class 2A carcinogen shows adequate evidence of carcinogenicity in experimental animals but limited evidence of this in humans; however, there is strong evidence that the mechanisms of carcinogenesis which function in experimental animals also operate in humans. B[a]P is formed as a by-product of the combustion of organic materials [21]. B[a]P has been found in the air, water, soil, food supply, and in cigarette smoke [22]. Although most studies have successfully used DMBA as the carcinogen in the cheek pouch it is not environmentally relevant. Benzo[a]pyrene had been used by Salley in the mid 1950's but he found it to be a weak carcinogen [14]. Solt tried several carcinogens in 1987 including B[a]P, however, after 40 weeks of 0.5% B[a]P treatment there were no lesions [23]. The B[a]P doses used by Salley and Solt were the same doses used for DMBA treatments. More recently, Ashurst produced tumors in mouse skin using higher doses of B[a]P. The doses were increased approximately 100 times that of the typical DMBA dose [24]. Using this information, Gimenez-Conti developed a protocol to produce squamous cell carcinomas in the hamster cheek pouch using B[a]P as the carcinogen (Brandon, Gimenez-Conti, in progress). Using 2% B[a]P and a complete carcinogenesis protocol, diagrammed in Figure 1, pre-malignant lesions are produced at 16 weeks and squamous cell carcinomas at 20 weeks. Figure 2 shows an inverted normal cheek pouch.

It is thin, transparent tissue with the muscle collecting in the center of the pouch. Figure 3 is an inverted cheek pouch treated with 2% B[a]P for 32 weeks. Notice the increased tissue density and the three obvious lesions shown by the arrows. Figures 4a and 4b are two premalignant lesions produced by 20 weeks of 2% B[a]P treatment. These lesions show hyperkeratosis, hyperplasia, and dysplasia. In human, many of these lesions are reversible. Figure 5 is a carcinoma in situ produced after 32 weeks of 2% B[a]P treatment. It appears very similar to a squamous cell carcinoma but the basement membrane has not yet been penetrated. This lesion is irreversible. Figure 6 is a squamous cell carcinoma in the hamster cheek pouch which was produced after 32 weeks of 2% B[a]P treatment. The microinvasions into the connective tissue, the displaced keratin pearls throughout the lesion, the dysplasia and anaplasia, as well as the multiple mitotic cells are diagnostic markers used to analyze squamous cell carcinomas. The results of these previous studies have established B[a]P as an applicable carcinogen for further developmental and chemoprevention studies with the hamster cheek pouch model.

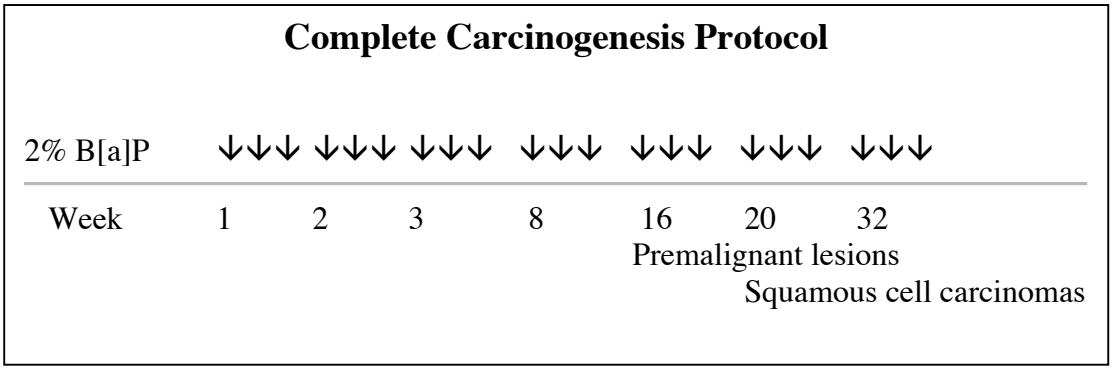


Figure 1. Complete carcinogenesis protocol. Induction and progression of oral squamous cell carcinomas in the hamster cheek pouch using 2% B[a]P suspended in mineral oil and a complete carcinogenesis protocol.

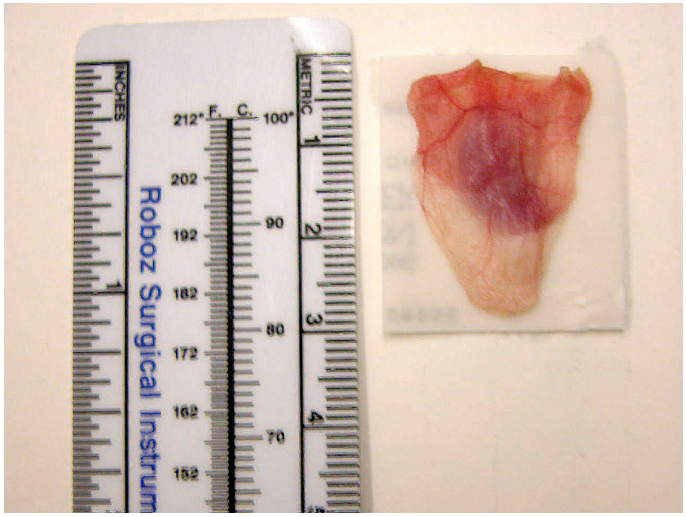


Figure 2. Inverted normal cheek pouch.

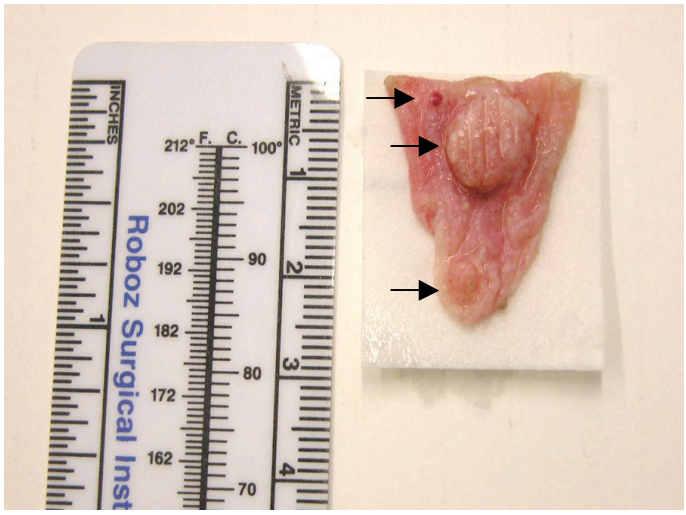
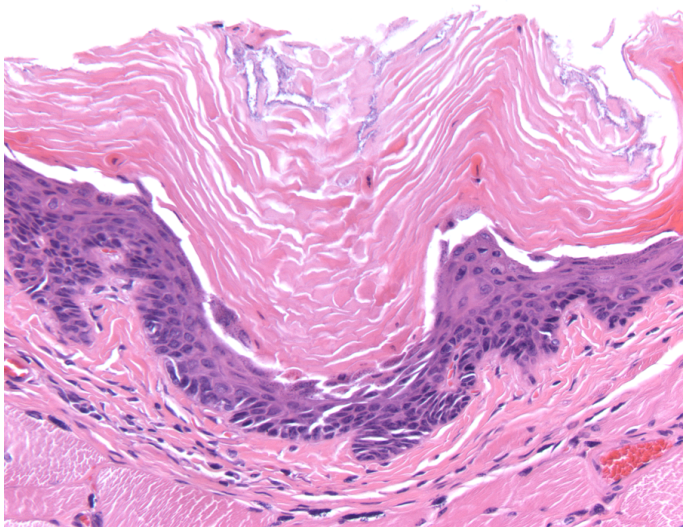


Figure 3. Inverted B[a]P cheek pouch. Cheek pouch treated with 2% B[a]P for 32 weeks.

a.



b.

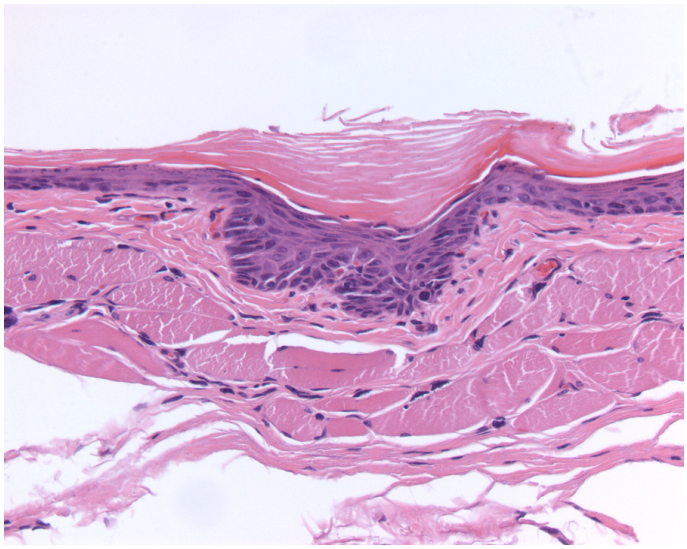


Figure 4. Histopathology of premalignant lesions. Histopathology (a. and b.) of premalignant oral lesions in the hamster cheek pouch treated with 2% B[a]P for 20 weeks (20X).

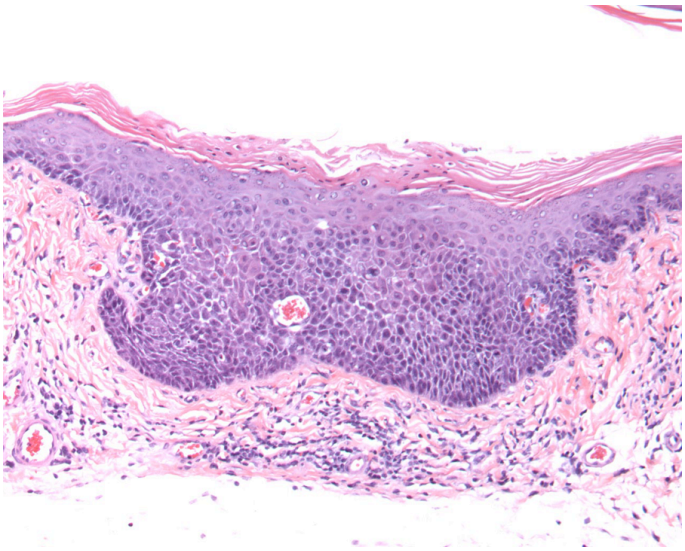


Figure 5. Histopathology of carcinoma in situ. Carcinoma in situ in the hamster cheek pouch treated with 2% B[a]P for 32 weeks (10X).

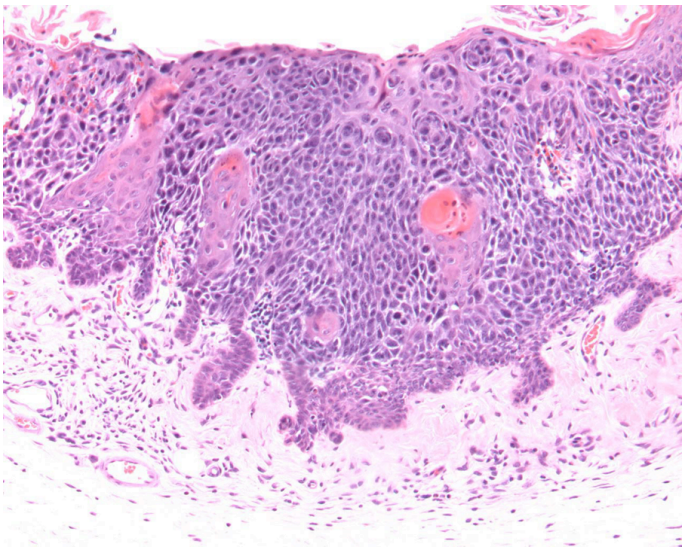


Figure 6. Histopathology of squamous cell carcinoma. Squamous cell carcinoma of the hamster cheek pouch treated with 2% B[a]P for 32 weeks (10X).

Chemoprevention

Chemoprevention is defined as the systemic use of natural or synthetic agents to reverse or even suppress the progression of a premalignant lesion [25]. As many as 500 agents have been recognized as possible chemopreventive agents [26]. According to Linger there are three approaches to oral cancer prevention: 1. patient education, 2. early clinical detection of premalignant lesions, and 3. the use of chemopreventive agents [27]. Many known chemopreventive compounds are abundant, inexpensive and easily accessible. They are often used around the world as every day dietary ingredients. Two very important aspects to look for when searching for chemopreventive compounds is to find natural or synthetic agents that will have minimal toxic side effects and that can be easily taken by people at high risk for malignancies. However, the use of many of these compounds has been hampered due to toxic side effects after long-term exposure or because of high doses.

When studying oral cancer it is of particular importance that these compounds be able to stop the growth and transformation of premalignant lesions such as leukoplakia from progressing into squamous cell carcinomas. Most chemopreventive agents can be divided into three categories depending on their point of inhibition: compounds preventing formation of carcinogen from precursor compounds, blocking agents, or suppressing agents. The first category describes the compounds that may inhibit initiation. Blocking agents function as a barrier preventing a carcinogen from interacting with the target tissue. Suppressing agents act to prevent the formation of neoplasia after a cell has been exposed to a carcinogen [28]. Some chemopreventive agents fit in more

than one of these categories. The two compounds chosen for this study, curcumin and green tea polyphenols, can prevent initiation or prevent the formation of carcinogens, and they both have been found to suppress developing neoplasms. Effective compounds like these are often inexpensive, show minimal toxicity, have easy treatment protocols, and are abundant worldwide. These compounds could lead to strategies necessary to derive ways to disrupt, regress, and prevent carcinogenesis from occurring.

Green Tea

Other than water, tea is the most widely consumed beverage in the world. 500,000 metric tons of dried green tea are manufactured every year. Green tea and black tea are made from the same raw tea leaves (Camellia sinensis). The manufacturing technology is what differentiates the two. The manufacturing of green tea limits the oxidative processes. Any oxidative transformations deteriorate quality of the tea [29]. Green tea is made by roasting or steaming fresh tea leaves in such a way that the chemical composition changes minimally. The tea is then sweated to achieve tenderness, and rolled. It is then dried to remove moisture, and rolled again to crush the leaf blades and stalks. The tea is cooled and sifted to prepare for firing. Firing the tea decreases the moisture content to 3-5% so the tea can then be sorted and packaged [29]. This process limits chemical transformations and only reduces the polyphenols and catechins by a minimal 17-18% [29]. Listed in Table I are some of the major components that make up dried green tea leaves.

Table I. Composition of green tea leaf [30]

Polyphenols	36.0%
Carbohydrates	25.0%
Protein	15.0%
Lignin	6.50%
Ash	5.0%
Amino Acids	4.0%
Methyl xanthines	3.50%
Lipids	2.0%
Organic Acids	1.50%
Chlorophyll	0.50%
Carotenoids	<0.10%
Volatiles	<0.10%

Catechins comprise approximately 30% of the weight of extract solids of manufactured green tea, this compared to 3-10% of the weight of extract solids of manufactured black tea. These catechins are believed to have anti-oxidation properties, antimicrobial properties, and anti-carcinogenic properties. Table II lists the isolated catechins found in the fresh tea leaf.

Table II. Principal leaf catechins. Principal leaf catechins measured in % dry weight [30].

(+) Catechin	1-2%
(-) Epicatechin (EC)	1-3%
(-) Epicatechin gallate (ECG)	3-6%
(+) Gallocatechin	1-3%
(-) Epigallocatechin (EGC)	3-6%
(-) Epigallocatechin gallate (EGCG)	7-13%
Total catechins	16-30%

It is believed that the chemopreventive activity of green tea is due to the anti-oxidative and possible anti-proliferative effects of these catechins [29]. Reactive oxygen

species can damage DNA, alter gene expression or affect cell growth and differentiation [31]. These catechins could bind to the cell inhibiting the generation of reactive oxygen species [32], or they may scavenge reactive oxygen species, thus, preventing DNA damage [33]. Other possible mechanisms including anti-pyretic, diuretic, anti-inflammatory, radioprotective and antihepatotoxic activities [29] [32] have also been demonstrated. These polyphenols have inhibited skin tumor initiation by interacting with Benzo[a]pyrene diol epoxide (BPDE), the ultimate cancer causing metabolite of B[a]P, preventing the formation of DNA adducts and therefore preventing initiation and tumorigenesis [34]. They have also been shown to inhibit lipoxygenase and cyclooxygenase activities thus inhibiting cell proliferation [35]. Green tea may also play a role in inducing apoptosis [36], and recently it has been shown to inhibit angiogenesis by inhibiting the growth of endothelial cells [37]. In other *in vivo* experiments, papilloma-bearing mice were given green tea as their sole source of drinking fluid and/or they were injected with the green tea polyphenols three times per week. The green tea inhibited the growth of the established papillomas, and some tumors were shown to regress [38]. Other studies have shown that oral administration of green tea inhibits the progression of papillomas to carcinomas [39], and inhibits lung tumorigenesis [40]. Inhibition of carcinogenesis by green tea polyphenols has also been demonstrated in many other models including esophagus, stomach, liver, duodenal, small intestine, pancreas, colon, bladder, prostate and mammary gland cancers [41]. Due to the wide spread popularity of green tea consumption it could prove to be a tremendous asset in chemoprevention.

Curcumin

Curcumin, an extract of tumeric, comes from the rhizome of the Curcuma longa Linn plant. This plant is primarily cultivated in South-eastern Asia. At maturity the rhizome, or root, is harvested, dried, and ground into powder. Commercially available curcumin contains 77% curcumin, 17% demethoxycurcumin, and 3% bisdemethoxycurcumin (the latter two being considered curcuminoids) [42]. It has been considered a traditional medicine and used for centuries to treat inflammatory diseases [43]. In addition, it is also known to possess strong antioxidant activities [44]. The use of curcumin dates back as early as the 7th century AD in traditional Chinese medicine. It was used to treat fever, diarrhea, bronchitis, parasitic worms, leprosy, and kidney and bladder inflammation. It has also been mixed with hot water to be used as a cold remedy. Some herbalists have suggested inhalation of burning curcumin fumes to relieve coughs, and topical applications to bruises, eye infections, leech bites, and other skin infections or conditions. Recent reports found that it also holds anti-carcinogenic actions [45,46]. It has been shown to inhibit skin tumors in mice [47], forestomach, duodenal, and colon cancers in mice [48], and reduce tumor incidence in radiation-induced tumor initiation in rat mammary gland [49]. Curcumin was found to inhibit cell progression in normal, dysplastic, and malignant human oral epithelial cell lines [50]. It is effective against both the initiation and promotion stages of carcinogenesis [45,47,51]. The mechanisms of this compound are not completely understood. There have been many publications verifying different mechanisms curcumin uses to inhibit tumorigenesis. When applied topically to mouse skin it inhibits B[a]P-DNA adduct

formation, therefore inhibiting initiation [45]. In *in vitro* studies using human oral squamous cell carcinoma cells, curcumin inhibits the activation of cytochrome P-450, a primary carcinogen-activating enzyme necessary for B[a]P metabolism, thus preventing B[a]P metabolism and preventing initiation [52]. Curcumin has also been reported to inhibit DNA adduct formation [53]. Anti-promotional effects include its ability to inhibit cyclooxygenase and lipoxygenase activities therefore inhibiting cell proliferation [54]. It has also been reported to enhance apoptosis [55]. Curcumin has been shown to inhibit the activation of NF-κB, a transcription factor that when activated induces inflammation and cellular adhesion molecules necessary for invasion and metastasis [56]. Western medicine has recently taken a great interest in curcumin and its anti-carcinogenic activities. Much research has been done and much more is needed to determine mechanisms of action used by this popular compound. Due to its extreme popularity and wide use throughout the world as a spice, a food preservative, and a coloring agent for foods and drugs, it could prove to be a valuable, inexpensive, and easily accessible chemopreventive agent for oral cancer.

MATERIALS AND METHODS

Chemicals

Curcumin, Benzo[a]Pyrene, Mineral Oil, and 5-bromo-2-deoxyuridine (Brdu) were all purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Green Tea Polyphenols was obtained from LKT Laboratories, Inc. (St. Paul, MN, USA).

Animal Treatment

50 4-5 week old male Syrian hamsters were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). The animals were housed four per cage in climate-controlled quarters (55% ±5 humidity, 72^{O}F±2, and spring cycle with 14 hours of light and 10 hours of dark) with woodchip bedding. They were provided food and water ad libitum. The animals were quarantined for three weeks and tested for hamster viruses and parasites. Following the three weeks of quarantine each animal began treatments with the appropriate chemopreventive compound suspended in mineral oil followed thirty minutes later by 2.0% B[a]P also suspended in mineral oil. The right cheek pouch of each hamster was painted topically with the compounds using a camel hair paintbrush. Animals were treated three times per week for eight weeks. At the end of eight weeks the animals were injected intraperitoneally with BrdU at a dose of 50mg/kg of body weight to identify proliferating cells. The animals were then sacrificed at 24 hours or 72 hours after the last treatment.

Tissue Processing and Sectioning

The right cheek pouch of each animal was harvested, grossly analyzed, then rolled into a ball and placed in 10% neutral buffered formalin. The samples were fixed for seven days and then processed. The processing consisted of one hour in 70% ethanol, two changes one hour each in 95% ethanol, three changes one hour each in 100% ethanol, two changes one hour each in hemo-de (xylene substitute), and two changes one hour each in 60º Celsius (low melting point) paraffin. The spherical hamster cheek pouches were cut in half and embedded cut side down in low melting point paraffin. Ten four-micrometer sections were cut using a rotary microtome. The sections were placed on a 45º Celsius waterbath to remove wrinkles and then picked up onto poly-L-lysine (PLL) treated slides. One slide of each sample was put into a 60º Celsius oven for twenty minutes to adhere the tissue to the slides, and then stained with hematoxylin and eosin for further histological evaluation.

Immunohistochemistry

Anti-Brdu: Four-micrometer sections on PLL slides were placed into a 60º Celsius oven for twenty minutes to adhere the tissue to the slides. Once cooled the slides were deparaffinized and rehydrated by submerging slides into two changes of hemo-de for five minutes each, two changes of 100% ethanol for five minutes each, one change of 95% ethanol for five minutes, then into distilled water for five minutes. The slides were put into 3% hydrogen peroxide from Sigma-Aldrich Company in distilled water for fifteen minutes to block endogenous peroxidase then washed three times five minutes

each in Dulbecco's phosphate buffered saline (PBS) from Sigma-Aldrich Company containing 1% bovine serum albumin from Roche Diagnostic Corporation (Indianapolis, IN, USA). They were then put into 1N hydrochloric acid from Fisher Scientific (Fair Lawn, NJ, USA) at 37º Celsius for 20 minutes. Slides were washed as previously described. Slides were then incubated in 0.5% protease XXIV from Sigma-Aldrich Company for fifteen minutes then washed. Slides were incubated in 10% normal horse serum from Gibco BRL (Grand Island, NY, USA) in PBS for 30 minutes to block non-specific staining then, without washing, were incubated in mouse monoclonal Anti-Brdu from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA) at 1:150 for one hour at room temperature. The slides were then washed and incubated in Biotinylated Anti-Mouse IgG from Vector Laboratories (Burlingame, CA, USA) at 1:200 for thirty minutes at room temperature. The slides were washed and incubated in Avidin-Biotin Complex from Vector Laboratories for thirty minutes at room temperature. The slides were washed and developed with 3,3'-Diaminobenzidine (DAB) from Sigma-Aldrich Company. They were rinsed in distilled water and counterstained in Gill 1 hematoxylin from ThermoShandon (Pittsburgh, PA, USA) for one minute, rinsed in distilled water, air-dried and coverslipped with a synthetic resin permanent mounting medium Consul Mount also from ThermoShandon.

TUNEL: Four-micrometer sections on PLL slides were placed into a 60º Celsius oven for twenty minutes to adhere the tissue to the slides. Once cooled the slides were deparaffinized and rehydrated by submerging slides into two changes of hemo-de for five minutes each, two changes of 100% ethanol for five minutes each, one change of

95% ethanol for five minutes, then into distilled water for five minutes. The ApopTag Peroxidase In Situ Apoptosis Detection Kit from Serologicals Corporation (Norcross, GA, USA) was used for this assay. The slides were incubated in 5mg/ml proteinase K from Sigma-Aldrich Company for ten minutes and then washed in PBS. They were then put into 3% hydrogen peroxide in PBS for five minutes to block endogenous peroxidase. The slides were washed in PBS and incubated in the kit-provided equilibration buffer for no longer than thirty minutes at room temperature. The kit-provided TdT enzyme was mixed with the reaction buffer at 1:100 and incubated on the slides for one hour at 37° Celsius. The slides were washed with the kit-provided stop-wash buffer diluted in distilled water for fifteen minutes at room temperature. They were then washed and incubated in the kit-provided anti-digoxigenin peroxidase conjugate for 30 minutes at room temperature. The slides were washed, DAB was used to develop, and Gill 1 hematoxylin was used as the counterstain. The slides were then washed in distilled water, air dried, and coverslipped using synthetic resin for the permanent mounting medium.

Anti-P53: Four-micrometer sections on PLL slides were placed into a 60° Celsius oven for twenty minutes to adhere the tissue to the slides. Once cooled the slides were deparaffinized and rehydrated by submerging slides into two changes of hemo-de for five minutes each, two changes of 100% ethanol for five minutes each, one change of 95% ethanol for five minutes, then into distilled water for five minutes. The slides were put into 3% hydrogen peroxide in distilled water for fifteen minutes to block endogenous peroxidase and washed three times five minutes each in PBS containing 1% bovine

serum albumin. They were then boiled for fifteen minutes in 10mM citrate buffer 6.0 pH for antigen retrieval. After the slides cooled they were washed and incubated in 10% normal horse serum from Gibco BRL in PBS for 30 minutes to block non-specific staining, then without washing, were incubated in monoclonal Anti-p53 from Vector Laboratories at 1:100 overnight at 4^{O} Celsius. The slides were then washed and incubated in Biotinylated Anti-Mouse IgG at 1:200 for thirty minutes at room temperature. The slides were washed and incubated in Avidin-Biotin Complex for thirty minutes at room temperature. The slides were washed and developed with DAB. They were rinsed in distilled water and counterstained in Gill 1 hematoxylin for one minute, rinsed in distilled water, air-dried and coverslipped with a synthetic resin permanent mounting medium.

RESULTS

This short-term experiment was completed to determine if green tea and curcumin show any chemopreventive effects against B[a]P- induced oral carcinogenesis in the hamster cheek pouch using short-term mechanistic markers. Table III shows the division of the treatment groups, the number of animals per group, the average weights at the beginning of the experiment, the average weights at the end of the experiment, and the average daily gain.

Table III. Treatment groups and weights. Short-term experiment treatment groups, number of animals, average weights, and average weight gains.

Group	Treatment	Number of Animals	Week 1 Average Weight (grams)	Week 8 Average Weight (grams)	Average Daily Gain (grams)
I	Mineral Oil	8	110.6	120.7	0.180
II	2.0% B[a]P	8	108.3	116.8	0.152
III	2.5% Green Tea	8	135.5	145.5	0.179
IV	5.0% Green Tea	8	140	154.9	0.266
V	0.6% Curcumin	8	123.3	131.4	0.145
VI	6.0% Curcumin	8	137.3	146.5	0.164

At 7-8 weeks of age treatment began on all animals. All compounds were suspended in mineral oil. Only the right cheek pouch of each animal was treated topically using a paintbrush. Previous experiments in our lab determined that each dip of the paintbrush into the viscous mineral oil yielded approximately 200µl per application. Group I was treated one time per treatment with mineral oil, three treatments per week for eight weeks. Group II was treated one time per treatment with 2.0% B[a]P, three treatments per week for eight weeks. Group III was treated with 2.5% green tea polyphenols followed thirty minutes later by 2.0% B[a]P per treatment, three treatments per week for eight weeks. Group IV was treated with 5.0% green tea polyphenols followed thirty minutes later by 2.0% B[a]P per treatment, three treatments per week for eight weeks. Group V was treated with 0.6% curcumin followed thirty minutes later by 2.0% B[a]P per treatment, three treatments per week for eight weeks. Group VI was treated with 6.0% curcumin followed thirty minutes later by 2.0% B[a]P per treatment, three treatments per week for eight weeks.

At the end of the eighth week treatment was halted and animals were sacrificed. Half of the animals from each group were sacrificed 24 hours after the last treatment. This would allow us to look at the more acute cellular activities of the cheek pouch. The remaining half of the animals were sacrificed 72 hours after the last treatment to examine the more chronic cellular activities of the cheek pouch.

Histopathology

The first parameter analyzed was gross appearance at time of sacrifice. Results are illustrated in Table IV. All Group I negative control hamsters treated with mineral oil appeared to have normal pouches. The pouches were pink in color and showed no signs of irritation. Of the positive control Group II treated with 2% B[a]P suspended in mineral oil 75% of the animals had red, congested pouches while 25% of the pouches appeared normal. Of the experimental Group III animals treated with 2.5% green tea polyphenols + 2.0% B[a]P, 50% of them had red, congested pouches while the remaining 50% appeared normal. Of experimental Group IV animals treated with 5.0% green tea polyphenols + 2.0% B[a]P, 75% had only minor redness and irritation and 25% appeared normal. Of experimental Group V animals treated with 0.6% curcumin + 2.0% B[a]P, 100% of the animals appeared normal. Of experimental Group VI animals treated with 6.0% curcumin + 2.0% B[a]P 10% of the pouches had minor redness or irritation, and 90% of the pouches looked normal. The pouches were then fixed, processed, sectioned, and stained with hematoxylin and eosin (H&E). Using the H&E slides we were able to analyze the histology of each pouch and determine the presence of inflammation, hyperplasia, hyperkeratosis, dermis thickening, and/or congestion. Figure 7 demonstrates the epithelium of a normal pouch visualizing a thin layer of keratin at the surface. Just below the keratin layer are two to three layers of differentiating cells. Next, is a single layer of basal cells or proliferating cells. The dermis consists of loose fibrous connective tissue, and just below is a layer of muscle. Figure 8 is a typical pouch treated with 2% B[a]P having hyperkeratosis in the keratin layer, increased hyperplasia

in the differentiating cell layers (four to five layers of differentiating cells), and hyperplasia in the basal layer. The connective tissue of the lamina propria is more dense than normal, and the blood vessels appeared to be congested. The experimental groups showed slight hyperkeratosis and only minor hyperplasia and the dermis was normal.

Table IV. Gross appearance evaluation. Evaluation of gross appearance of the hamster cheek pouch.

Group	Treatment	Normal Appearance	% with mild irritation	% with severe irritation
I	MO	100%	----	----
II	B[a]P	25%	----	75%
III	2.5% GreenTea	50%	----	50%
IV	5.0% GreenTea	25%	75%	----
V	0.6% Curcumin	100%	----	----
VI	6.0% Curcumin	90%	10%	----

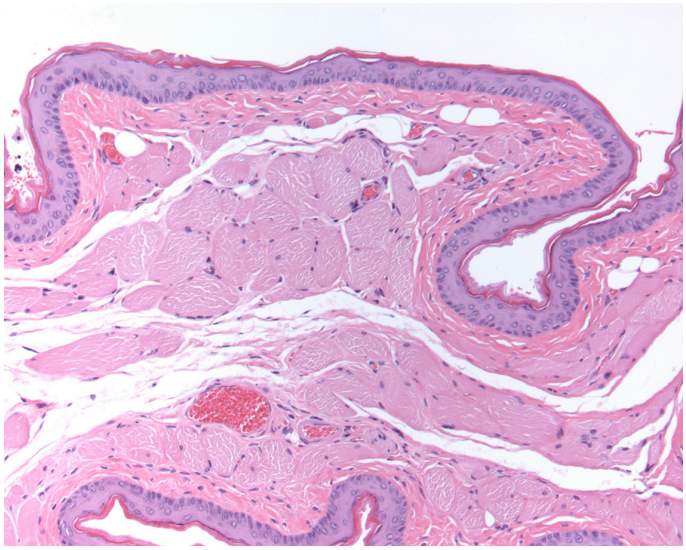


Figure 7. Histology of normal cheek pouch.

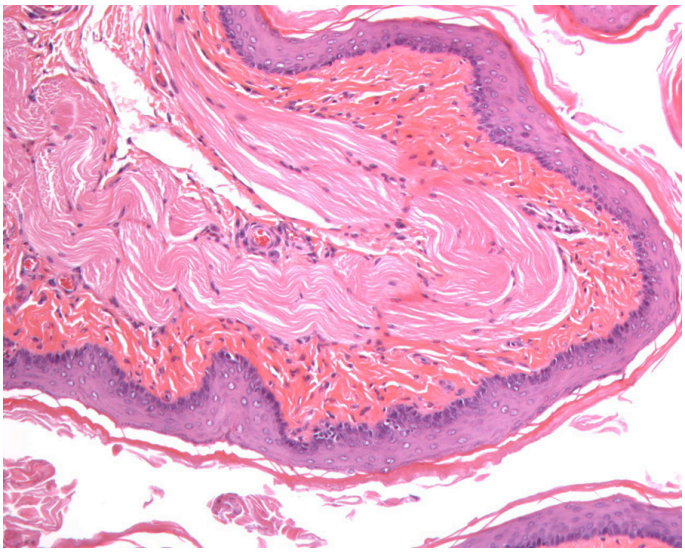


Figure 8. Histopathology of B[a]P cheek pouch. Histopathology of hamster cheek pouch treated with 2% B[a]P for 8 weeks.

BrdU Immunohistochemistry

Next step was to quantify cellular proliferation in the cheek pouches of the normal and experimental hamsters. Because cancer is associated with an increase in proliferation and/or a decrease in cell death losing the homeostatic balance between cell proliferation and cell death, determining proliferation rate is one of the most important biomarkers in cancer research. Before sacrificing the animals they were injected with Brdu, a DNA precursor that incorporates into the DNA of the cells cycling through the S-phase of the cell cycle.

These proliferating cells were detected by immunohistochemistry using an anti-Brdu antibody. The rate of proliferation was determined by counting a total of 600 basal cells in randomly selected fields. The proliferation index of normal squamous cell epithelium in the hamster oral mucosa is 5-8%. The rate of proliferation in the control pouches treated with mineral oil and sacrificed 24 hours after the last treatment was 11%. This was anticipated due to the acute irritation of the treatments. Expectedly, at the 72-hour sacrifice the rate of proliferation was back down to 8%. The proliferation rate at the 24-hour sacrifice in the positive control group treated with B[a]P was very high at 38%, and at the 72-hour sacrifice decreased to 14%. Figure 9 graphs out the experimental groups of green tea polyphenols compared with the control groups. Comparing the 24-hour groups with the positive control group we see a statistically significant decrease in proliferation in the two extreme doses of green tea, while the 72-hour results had only a slight increase in proliferation. Table V shows the percent inhibition of proliferation in each dose of green tea polyphenols. At the 24-hour time-

point the 2.5% green tea polyphenols had a 43.6% inhibition and the 5.0% green tea polyphenols had a 29.2% inhibition. There was no inhibition in the 72-hour time-point. Figure 10 graphs the groups treated with curcumin. Of these two doses the lesser dose significantly decreased the number of proliferating cells when compared to the positive control group. The greater dose of curcumin showed a slight decrease. At the 72-hour sacrifice only the lesser dose, 0.6% curcumin had a statistically significant decrease in proliferation. Table VI shows the percent inhibition of proliferation in each dose of curcumin. At the 24-hour time-point the 0.6% curcumin had 33.1% inhibition while the 6.0% curcumin had only an 8.6% inhibition. At 72-hours only the 0.6% dose had inhibition and it was a 52.4% inhibition.

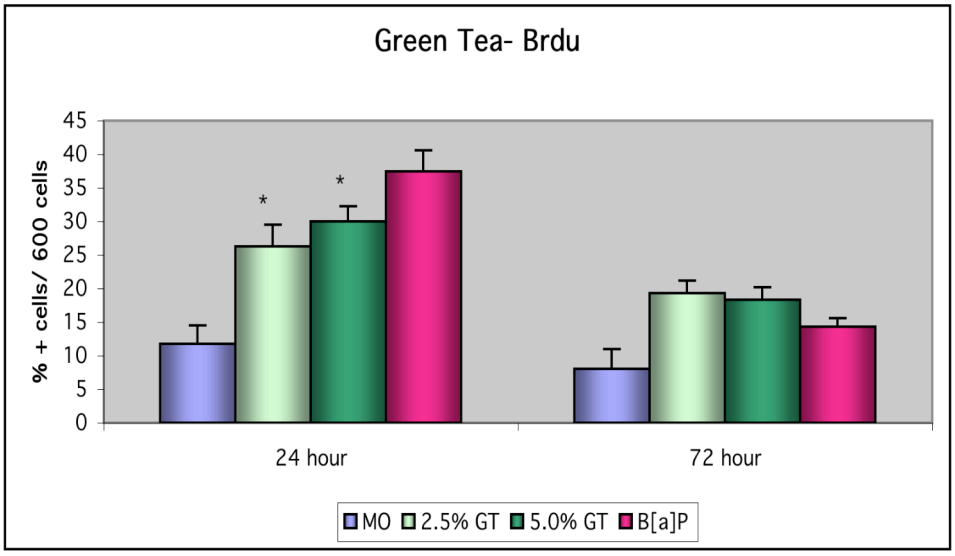


Figure 9. Green tea BrdU incorporation. BrdU incorporation in green tea treated cheek pouches. Represented are the group averages of percent positive cells per 600 cells counted from random fields.

Table V. Green tea proliferation inhibition. Percent proliferation inhibition on green tea treated pouches.

	24 Hour	72 Hour
2.5% Green Tea	43.6%	< 0%
5.0% Green Tea	29.2%	< 0%

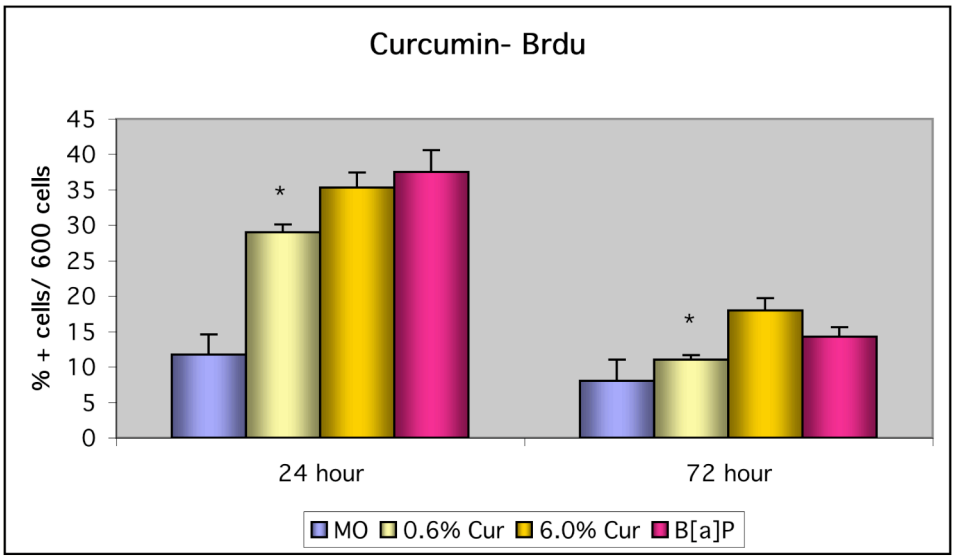


Figure 10. Curcumin BrdU incorporation. BrdU incorporation in curcumin treated cheek pouches. Represented are the group averages of percent positive cells per 600 cells counted from random fields.

Table VI. Curcumin proliferation inhibition. Percent proliferation inhibition in curcumin treated pouches.

	24 Hour	72 Hour
0.6% Curcumin	33.1%	52.4%
6.0% Curcumin	8.6%	< 0%

P53 Immunohistochemistry

The P53 gene has been considered the "guardian of the genome" by many researchers. It is considered to be a checkpoint factor, or an "emergency brake" in the cell cycle. It prevents damaged genetic information from being passed from generation to generation. It plays a major role in the transcription of DNA, in numerous metabolic processes and in cell growth and proliferation. When a mutation occurs in the p53 gene it loses its ability to block the abnormal cells from growing. P53 protein is in the nucleus, and if the DNA is damaged the protein concentration increases dramatically. High levels of p53 are an indication of possible DNA damage and usually will lead to cell cycle arrest or apoptosis. The loss of p53 function is found in many human and experimental cancers. Alterations in the p53 gene lead to genetic instability and excessive cellular growth [57]. The possible dysfunction or activation of p53 gene can be visualized by immunohistochemistry as an accumulation of p53 protein. It has been determined that p53 protein accumulation in the hamster cheek pouch carcinogenesis model is similar to that of human oral lesions, and is expressed in early phases of tumorigenesis [58].

The cellular accumulation of p53 protein was labeled using an anti-p53 antibody. The percent positive cells were calculated per 1000 cells. In the negative control groups, 24-hour and 72-hour, there was no expression of p53 protein accumulation. At the 24-hour sacrifice, the B[a]P treated group showed 15% of the cells positive for p53 protein accumulation. Figure 11 graphs the results of the green tea polyphenol treated pouches. The lesser dose, 2.5% green tea polyphenol was the only experimental group that had a

statistically significant decrease in p53 protein accumulation at the 24-hour sacrifice when compared to the positive control group. The 72-hour sacrifice showed no significant effect. Table VII shows the percent inhibition of p53 protein accumulation in each dose. At 24-hours the 2.5% green tea polyphenols had a 58.5% inhibition and the 5.0% green tea polyphenols had a 33.3% inhibition. At the 72-hour time-point only the 5.0% dose had inhibition and it was only a 20% inhibition. Figure 12 graphs the results of the curcumin treated pouches. At 24-hours both doses did have a lesser amount of p53 protein accumulation compared to the positive control group, however, these results were not statistically significant. Table VIII shows the percent inhibition of p53 protein accumulation in each dose. At the 24-hour time-point the 0.6% curcumin had a 49% inhibition and the 6.0% curcumin had a 21.1% inhibition. The 72-hour time-point had no inhibition of p53 protein accumulation.

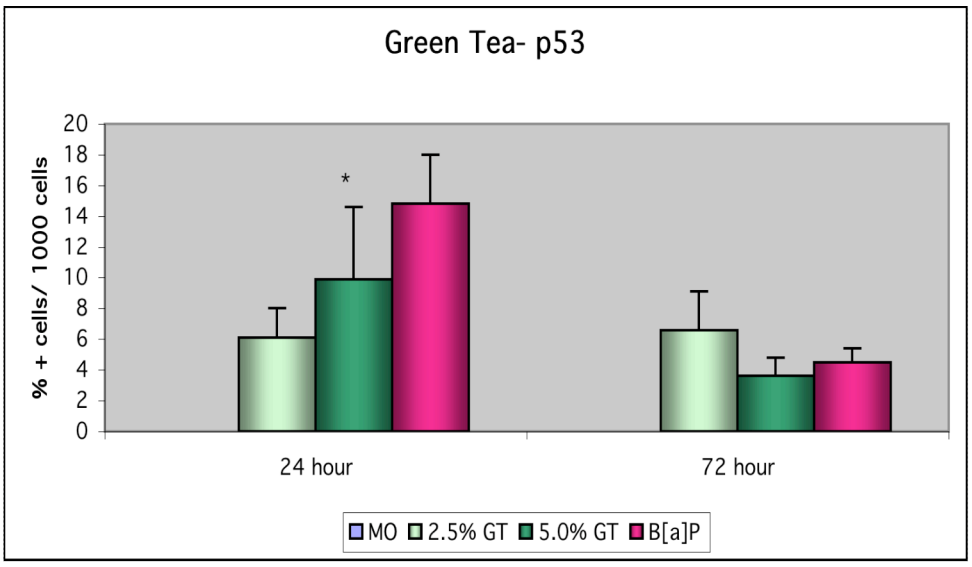


Figure 11. Green tea p53 protein accumulation. P53 protein accumulation in green tea treated cheek pouches. Represented are the group averages of percent positive cells per 1000 cells counted from random fields.

Table VII. Green tea p53 protein inhibition. Percent p53 protein accumulation inhibition in green tea treated pouches.

	24 Hour	72 Hour
2.5% Green Tea	58.5%	< 0%
5.0% Green Tea	33.3%	20.0%

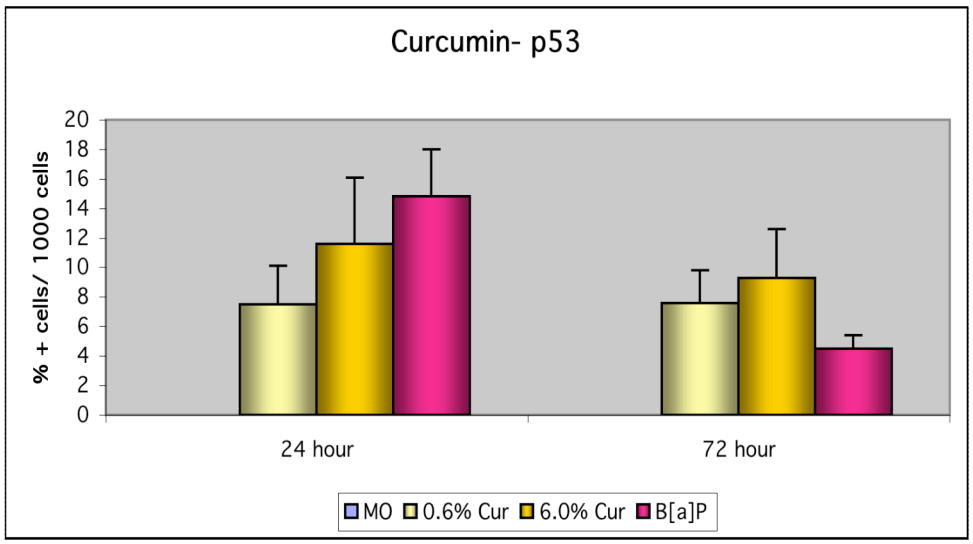


Figure 12. Curcumin p53 protein accumulation. P53 protein accumulation in curcumin treated cheek pouches. Represented are the group averages of percent positive cells per 1000 cells counted from random fields.

Table VIII. Curcumin p53 protein inhibition. Percent p53 protein accumulation inhibition in Curcumin treated pouches.

	24 Hour	72 Hour
0.6% Curcumin	49.0%	< 0%
6.0% Curcumin	21.1%	< 0%

Apoptosis Detection

Apoptosis is programmed cell death. It is also referred to as cellular suicide. It is a natural way for tissues to maintain homeostasis and to eliminate genetically damaged cells. Histologically, these cells shrink, the chromatin begins to degrade, the mitochondria break down and the cell breaks into small membrane-wrapped fragments to be phagocytized by macrophages and dendritic cells. Both curcumin and green tea are known inducers of apoptosis, although the exact mechanisms remain an enigma.

To detect the apoptotic cells we used the TdT-mediated X-dUTP nick end labeling (TUNEL) assay. Terminal deoxynucleotidyl transferase (TdT) is used to label double-stranded DNA breaks. The label is then detected with an anti-Digoxigenin and visualized with DAB. At the 24-hour sacrifice, the negative control group had approximately 2 apoptotic cells per 5 centimeters of epithelium, and the positive control group had approximately 5 apoptotic cells per 5 centimeters of epithelium. All of the experimental groups had only 4 to 6 apoptotic cells per 5 centimeters of epithelium, but neither group was statistically significant. However, the 72-hour sacrifice showed an increase in apoptosis in a dose dependent manner in both green tea polyphenols and curcumin. Figure 13 graphs the green tea polyphenol induced apoptosis showing a significant increase in the higher dose when compared to the negative control group. Figure 14 graphs curcumin-induced apoptosis showing a large increase, however, it was not stastically significant.

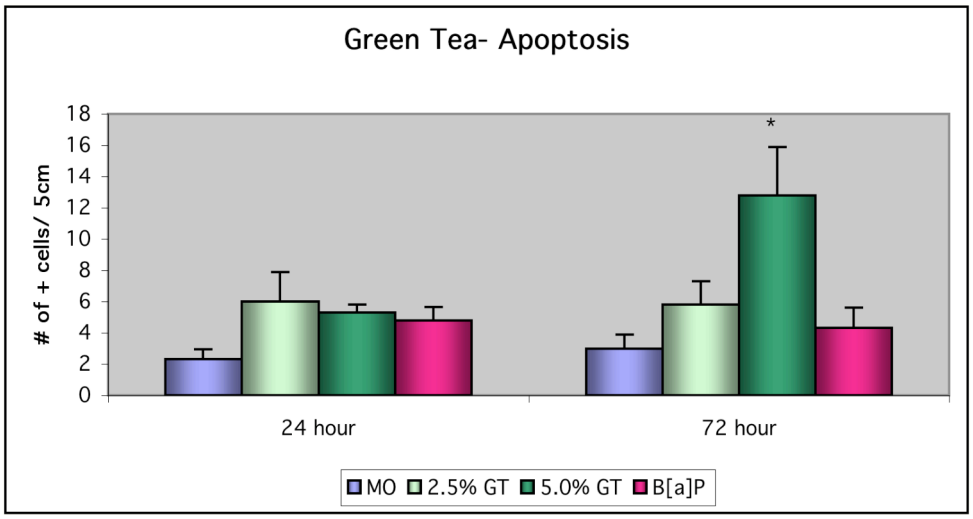


Figure 13. Green tea apoptosis activity. Apoptosis detection in green tea treated cheek pouches. Represented are the group averages of number of positive cells per five centimeters of epithelium.

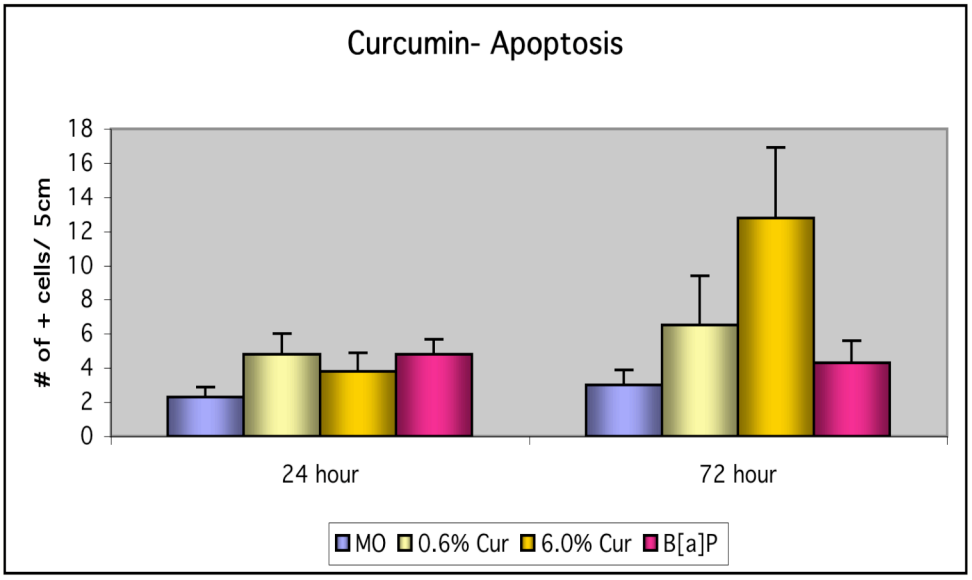


Figure 14. Curcumin apoptosis activity. Apoptosis detection in curcumin treated cheek pouches. Represented are the group averages of number of positive cells per five centimeters of epithelium.

DISCUSSION AND CONCLUSION

The present study, using short-term mechanistic markers, indicates that curcumin and green tea polyphenols may have plausible inhibitory effects on B[a]P-induced carcinogenesis in the hamster cheek pouch. Recent research has taken a great interest in environmental carcinogens. Many of the known carcinogens may be found in our air, soil, water, and food supplies. In particular, the environmental carcinogen, B[a]P [22], has been a contributor to cancers of respiratory and digestive origins [59-64]. Ruiz summarized that the epidemiologic evidence has shown an increased risk of oral cancer upon chronic consumption of alcohol and tobacco products [7], Weyand discussed both the induction of lung tumors and forestomach tumors by the ingestion of manufactured gas plant residues and B[a]P, respectively [59], and Rubin reviewed possible mechanisms of lung carcinogenesis in mice by inhalation of environmental tobacco smoke [64].

Curcumin and green tea polyphenols have been studies for centuries. They are well known for their antioxidant and anti-inflammatory activities and in more recent years for their anti-carcinogenic activities. Huang reviewed that topical treatment of curcumin inhibits B[a]P and DMBA initiation, TPA-induced tumor promotion, and epidermal cell proliferation. Administered through the diet of mice, curcumin reduced the number and the size of tumors, and inhibited duodenal tumors and colon tumors [65]. Green tea polyphenols administered orally have been shown to inhibit the growth of established papillomas, regress some papillomas [38] and inhibit papilloma progression

into carcinomas [39]. Mechanistically they inhibit DNA damage by scavenging reactive oxygen species [66], they prevent DNA adducts, therefore preventing initiation [34], and inhibit cell proliferation by inhibiting lipoxygenase and cyclooxygenase activities [35].

Because chemoprevention studies are often long and expensive, the use of early biomarkers could serve as valuable indicators in determining possible chemopreventive compounds by functioning as intermediate endpoints. Important early biomarkers include, but are not limited to, proliferation markers, P53 protein expression, and apoptosis detection. Previous data has shown that both green tea and curcumin may inhibit promotion [35,54] and initiation[34,53]. Promotion is vital stage of carcinogenesis where increased proliferation can allow the selection of mutated cells. This increased proliferation is often a precursor to carcinogenesis. Another early marker is P53 protein accumulation which often indicates possible p53 gene alterations [58], and high levels of apoptotic activity could indicate that the mutated cells are not being allowed to progress through the cell cycle and proliferate. The short-term biomarkers used in this experiment have determined that both green tea polyphenols and curcumin significantly inhibited carcinogen-induced proliferation, decreased the amount of cells expressing p53 protein, and induced apoptosis. Using these short-term mechanistic markers, our data has suggested that both green tea polyphenols and curcumin may have an inhibitory effect on B[a]P-induced carcinogenesis in the hamster cheek pouch. These results could suggest that these compounds may inhibit cell proliferation, a promotional effect, and/or prevent initiation by preventing DNA damage by blocking carcinogen metabolism and DNA adduct formation, or preventing genetic mutations. These results

do not prove that these compounds will inhibit the production of squamous cell carcinomas in our model, however, they do indicate that they may have a plausible chemopreventive effect.

It will be of great interest and importance to continue with long-term experiments to determine whether or not these compounds will actually inhibit the formation and progression of squamous cells carcinomas in the oral mucosa. It is also important to determine the mechanisms of action these compounds may use to carry out their chemopreventive effects. It is believed that they can inhibit *both* initiation and promotion of cells, reminding us of the complexity of these compounds and their possible mechanisms of actions. If research can recognize and prove the valuable effects of natural anti-carcinogenic compounds such as green tea and curcumin, particularly those that are commonly used throughout the world, there is possibility they can be used as beneficial tools against cancer simply by manipulating dietary intake to prevent carcinogen initiation or further progression into life threatening diseases. Chemoprevention is an important tactic in the fight against cancer, and could eventually lead to lower incidence rates, improve prognosis, and reduce mortality rates.

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Abstracts:

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