ANTICANCER RESEARCH *33*: 4757-4766 (2013)

# Chemoprevention of Oral Cancer by Lyophilized Strawberries

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**Abstract.** *Background/Aim: Oral cancer represents approximately 2.5% of all cancers in the United States, with five- and 10-year survival rates of 62% and 51%. In the present study, lyophilized strawberries (LS) were evaluated for their potential to inhibit tumorigenesis in the hamster cheek pouch (HCP) model of oral cancer and for their ability to modify expression of several genes relevant to oral cancer development. Materials and Methods: HCPs were painted three times a week for six weeks with 0.2% 7,12-dimethylbenz(a)anthracene (DMBA). Hamsters were given 5% or 10% LS in their diet prior to, during, and after, or only after carcinogen treatment. Animals were sacrificed 12 weeks from the beginning of DMBA treatment and the number of total lesions and tumors was determined. Results: A significant difference ($p<0.01$-$0.04$) in the number of tumors was found between the LS-treated groups and the carcinogen controls. Histological examination of HCPs revealed a significant reduction in mild and severe dysplasia following 12 weeks of treatment with LS. Molecular analysis revealed that genes related to tumor development were modulated by LS. Conclusion: These experiments support previous studies in HCP that demonstrated a chemopreventive activity by black raspberries and show, to our knowledge for the first time, that strawberries can inhibit tumor formation in an animal model of oral cancer.*

Oral cancer is the sixth most common type of cancer in the world (1-3) and represents approximately 2.5% of all cancer in the United States (4). For all stages, one-year survival rate is approximately 84%, with five- and 10-year survival rates of 62% and 51%, respectively, which have slowly declined over the past three decades (4). The overall survival rate is one of the lowest among the major sites of cancer and may be attributed to cancer tending to be diagnosed at advanced stages and a general lack of knowledge in the general population concerning the risks and signs of oral cancer (5). In addition, recurrence is a problem since 20% of patients may exhibit locoregional recurrence within two years' post-surgery and 22-42% of oral cancer patients will present with a second primary tumor within 5-8 years (6, 7). Major risk factors for oral cancer in the United States are the use of tobacco and alcoholic beverages, which may account for ~75% of all oral cancer (8, 9, 10).

Epidemiological studies have shown a strong correlation between the consumption of fresh fruits and vegetables and a decreased risk for oral cancer (11). In support of these observations, a series of pre-clinical investigations have demonstrated that black raspberries (*Rubus occidentalis*) prevent development of chemically-induced oral, esophageal, and colon cancer (12, 13, 14) in rodent models and Carlton *et al.* (15) showed that strawberries (*Fragaria* × *ananassa*) inhibit esophageal cancer in rats. Strawberries are of interest due to their ability to be cultivated in most parts of the world and their large content of bioactive components with potential chemopreventive activity, including vitamins A, C, and E; folic acid, calcium, selenium, β-sitosterol, ellagic and ferulic acids; flavonols such as kaempferol and quercetin; and multiple anthocyanins (16). Preliminary evidence that components of strawberries may have chemopreventive potential originates from the findings that strawberry extracts inhibit the proliferation of human cancer cells and induce apoptosis *in vitro* (17, 18, 19, 20) and inhibit activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) in cultured cells (21). The pharmacological and chemopreventive activity of individual components of various fruits are reviewed by Stoner and Casto (22) and by Seeram (23). Based on literature data and results with black raspberries and strawberries in rodent models of aerodigestive tract cancer, we postulated that dietary administration of strawberries would inhibit the occurrence of malignant oral lesions and modify expression of genes related to the development of oral cancer.

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*Key Words:* Chemoprevention, oral cancer, strawberries, hamster cheek pouch.

Squamous cell carcinoma (SCC) of the oral mucosa is an excellent candidate cancer for assessment of chemoprevention: lesions are amenable to oral delivery of chemopreventive agents, effects can be visually monitored during treatment, and modulation or inhibition of genes or gene products involved in oral SCC constitute molecular targets against which chemopreventive approaches can be tested and validated. The Syrian hamster cheek pouch (HCP) has been used since 1954 as a model for oral cavity cancer (24) and for studies on inhibition of oral cancer by numerous chemopreventive agents. More than 60 articles have been published in which the HCP model has been used to follow the early development of oral cancer (25-28), to identify and characterize some of the genetic alterations occurring early and late in the progression of oral SCC (29-32), and to determine the effectiveness of various food components as anticancer agents (33). Many of the morphological and physiological characteristics that are seen in human oral SCC are also evident in chemically-induced HCP tumors (34, 35). There is a close histological resemblance between HCP tumors and human oral SCC and similar molecular events are present in both human and hamster oral SCC that may serve as viable biomarkers (36). As with human oral cancer, some tumors that develop in the HCP begin as verrucae or papillomas that become malignant and invasive, while others evolve from relatively flat dysplastic lesions comparable to leukoplakias in human oral mucosa (24, 28). In addition, chemically-induced HCP tumors exhibit many of the same genetic events that are present in human oral SCC (29-32, 36).

In the present study, lyophilized strawberries (LS) were evaluated for their potential to inhibit 7,12-dimethylbenz(a) anthracene-induced tumorigenesis in an established HCP model of oral cancer and for their ability to modify the expression of several genes relevant to oral cancer development. In addition, the temporal relationship between initiation and progression events and inhibition by LS was determined by their administration after formation of carcinogen-induced hyperplasia/dysplasia in order to mimic conditions found in the oral mucosa of current and former tobacco users.

## Materials and Methods

*Animals*. Male Syrian Golden hamsters (*Mesocricetus auratus*), 3-4 weeks of age, were obtained from the Charles River Laboratories (Wilmington, MA, USA). Three animals each were placed in plastic bottom cages with hardwood chip bedding and acclimated for one week. Food (AIN-76A pellets, a modified semi-synthetic diet; Dyets Inc., Bethlehem, PA, USA) and water were given *ad libitum*. Animals were maintained in filtered cages with an automatic watering system and were weighed bi-weekly during LS and carcinogen treatment. All experimental conditions were in accordance with NIH Guidelines and with protocols approved by The Ohio State University Animal Care and Use Committee (Protocol #2005A0113-1).

*Chemicals*. DMBA was obtained from Sigma-Aldrich (Milwaukee, WI, USA) and dissolved at a 0.2% concentration in dimethylsulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA, USA).The dosing solutions were allocated at 1.5 ml into 13×100 disposable tubes and stored at 4˚C in foil-wrapped racks. At each dosing, freshly-thawed carcinogen preparations were used.

*Diet*. Strawberries (*Fragaria* × *ananassa*) were supplied by the California Strawberry Commission (Watsonville CA, USA) as a lyophilized strawberry powder (LS) following freeze-drying by Van Drunen Farms (Momence IL, USA); the composition of the strawberry powder from this source has been reported by Stoner *et al*. (37). The LS powder was incorporated into AIN-76A pellets at 5% and 10% concentrations by weight (Dyets Inc., Bethlehem, PA, USA).The amount consumed per week per hamster on a diet of 10% strawberry powder is equal to human consumption of one cup of fresh strawberries per day. In order to maintain equivalent caloric conditions, the cornstarch content of the diet was reduced to account for the LS powder. LS-containing and control pellets were distributed into the cage feeding trays using approximately 200 g per cage. During a two-week period, additional pellets were added where needed. Complete changes of diet were conducted every 10-14 days with pellets stored at 4˚C. At this time, the residual pellets were weighed and the weight subtracted from the total amount added to each cage to give the amount of feed consumed per cage over the period.

*Induction of tumors and chemoprevention protocols*. Complete chemoprevention bioassay (CCB). After acclimation, three groups of 21 hamsters, 4-5 weeks of age, were fed pellets containing either 0%, 5% or 10% LS (Table I). Ten days later, the cheek pouches were treated with DMBA. Hamsters were lightly anesthetized and the pouch made accessible by inserting a small retractor at the side of the mouth and gently pulling laterally away from the hamster to expose the interior surface of the pouch. Both surfaces of each pouch were painted three times weekly for six weeks with a 0.2% solution of DMBA dissolved in DMSO using a no.4 sable hair brush. Animals in groups 1, 2, and 3 received AIN-76A pellets with 10% LS, 5% LS, or control pellets, respectively. Tumors of sufficient size in the control group (≤10 mm in greatest length) appeared at 11-12 weeks after beginning DMBA treatment. At 12 weeks, animals were euthanized by $CO_2$ asphyxiation and cervical dislocation, the cheek pouches were everted, and total lesions (leukoplakias, papillomas, and tumors) were enumerated. All evaluations of lesions and tumors were performed by two and often three observers. Tumors were measured in two dimensions (L×W), excised, and large tumors bisected with one aliquot placed in buffered formalin for 10-12h and the other aliquot snap-frozen in liquid nitrogen. The remaining cheek pouch with the tumors removed was divided equally into two halves and one section was immediately frozen in liquid nitrogen and stored at –80˚C. A second portion of the pouch was fixed in 10% neutral buffered formalin for no more than 12h and paraffin embedded in separate paraffin blocks. The HCP tissues that were fixed in formalin were sectioned on edge, hematoxylin and eosin (H&E)-stained, and evaluated histologically by an oral pathologist (BA); the frozen tissues/tumors were analyzed for gene expression.

*Post-initiation chemoprevention bioassay (PCB)*. HCPs were painted with 0.2% DMBA in DMSO three times-a-week for six weeks as outlined in Table I. Beginning 48 h after the last DMBA treatment, hamsters were administered AIN-76A pellets containing 10% (group

Table I. *Carcinogen initiation and chemoprevention protocol in hamster cheek pouch (HCP).*

| | Week | | | |
|---|---|---|---|---|
| Group | –2-0[a] | 0-6[b] | 6-12[c] | 12[d] |
| 1 (N=21) | 10% LS | DMBA 3×/week+10% LS diet | 10% LS diet | Lesions counted |
| 2 (N=21) | 5% LS | DMBA 3×/week+5% LS diet | 5% LS diet | Lesions counted |
| 3 (N=21) | Control diet | DMBA 3×/week+control diet | Control diet | Lesions counted |
| 4 (N=21) | Control diet | DMBA 3×/week | 10% LS diet | Lesions counted |
| 5 (N=21) | Control diet | DMBA 3×/week | 5% LS diet | Lesions counted |
| 6 (N=11) | Control diet | Control diet | Control diet | Harvest HCP |

[a]Hamsters in groups 1 and 2 were given AIN-76A pellets with 10% or 5% lyophilized strawberries (LS) or AIN-76A pellets (group 3) for 10 days prior to DMBA treatment. During this time, hamsters in groups 4, 5 and 6 were given AIN-76A pellets (Control diet) only. [b]Hamsters in groups 1-5 were treated 3×/week for six weeks by painting both surfaces of pouches with a no. 4 sable hair brush containing 0.2% DMBA in DMSO. [c]Beginning 48h after the last carcinogen treatment, hamsters in groups 4 and 5 received 10% or 5% LS pellets; groups 1 and 2 continued to receive LS pellets; and Groups 3 and 6 continued to receive AIN-76A pellets only. [d]At week 12, hamsters were euthanized by $CO_2$ asphyxiation and cervical dislocation. Total HCP lesions were enumerated (tumors, leukoplakias, papillomas). Tumors were measured, resected, divided into two equal sections, and half of each tumor was placed in 10% buffered formalin and half frozen in liquid nitrogen. Cheek pouches were excised, split longitudinally, and preserved as above.

4) or 5% (group 5) LS; the DMBA control animals (group 3) were treated with carcinogens at the same time as those in the accompanying CCB (Table I). A non-treated sentinel control group (group 6) received neither LS nor DMBA. Feeding and weighing schedules were the same as those used in the CCB and cheek pouches were harvested six weeks after commencement of LS treatment. The cheek pouches from all animals were everted, lesions and tumors enumerated, and all tumors measured and harvested as outlined above. The remaining cheek pouches were cut longitudinally after the resection of the tumors and tumors and cheek pouch halves fixed in formalin or snap-frozen in liquid nitrogen.

*Histological evaluation*. Serial 4 μm sections were cut on edge from formalin-fixed pouches and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA). H&E-stained slides of each HCP were prepared and total tissue sections from each animal were scanned at ×100 magnification by an oral pathologist. Each field was enumerated for the total number of four histological categories: normal epithelium, low-grade (mild) dysplasia, high-grade (severe) dysplasia, or carcinomas-*in-situ* (CIS). The classification scheme utilized was modified from criteria developed by Pozharisski *et al*. (38).

*Isolation of RNA and determination of RNA quality*. Total cellular RNA was isolated from frozen tissues following homogenization using a Tissue-Tearor rotor/stator type tissue homogenizer (Biospec Products, Inc., Bartlesville, OK, USA) and Qiashredder homogenization column (Qiagen, Valencia, CA, USA), in appropriate buffer using the RNeasy Mini Kit (Qiagen). Contaminating DNA was removed from isolated RNA *via* DNase digestion using a Turbo *DNA-free* kit (Ambion, Austin, TX, USA). RNA was quantified using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Capillary electrophoresis fluorescence was used to characterize RNA quality and size distribution using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). A quantitative measurement of RNA integrity was determined using the RNA integrity number (RIN) calculated with 2100 Expert Software™ (Agilent Technologies). RNA with RIN values greater than 9.0 were used for relative real-time reverse transcription PCR (rRT-PCR).

*Relative real-time reverse transcription PCR analysis*. First-strand synthesis of cDNA was performed using the High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA,USA) from 500 ng of total cellular RNA. Custom hamster-specific TaqMan Gene Expression Assays (Applied Biosystems) were designed from existing *Mesocricetus auratus* GenBank submissions. rRT-PCR was performed using hamster gene-specific primer/probe mixtures on a 7900HT fast Real-Time PCR System (Applied Biosystems). Hamster genes for analysis included: *β-actin* control (*Actb*), *p16*, *p13$^{Arf}$*, *p53*, cyclin B2 (*Ccnb2*), B-cell CLL/lymphoma 2 (*Bcl2*), bcl-2-like protein 4 (*Bax*), cyclin-dependent kinase 2 (*Cdk2*), cellular homologue of the avian myelocytomatosis virus oncogene (*c-Myc*), and vascular endothelial growth factor (*Vegf*) (target genes). The ΔΔCt analysis method (39) and the $2^{-\Delta\Delta CT}$ method (40) were used to determine relative gene expression levels of biomarker mRNAs.

*Statistical analysis*. Analysis of body weights and food consumption was performed using the generalized estimating equations (GEE) technique to accommodate the correlation of weights at different time points for each animal. For analyzing lesion counts, a log linear model with the treatment group as the single covariate was fitted to compare the mean lesion and tumor counts at different dose levels.

For gene mRNA expression, the $2^{-\Delta\Delta C_T}$ method (40) was used to analyze the rRT-PCR data. To estimate the amount of the target gene in DMBA-only treated cheek pouches (Group 3), expression levels were normalized to an endogenous reference gene (Actb) and compared to the untreated normal cheek pouches (untreated control group 6). A linear regression model was fitted with $C_T$ (threshold cycle number) as the response variable and treatment groups and gene group as covariates. Gene groups were coded as 1 if the gene was a target gene and as 0 if the gene was a reference gene; the model also adjusts for the cheek pouch effect. The interaction between treatment group and gene group was interpreted as:
$\Delta\Delta C_T=(C_{T,treat,tar}-C_{T,treat,ref})-(C_{T,control,tar}-C_{T,control,ref})$, where $C_{T,treat,tar}$ is the threshold cycle number of a target gene of the treated group and $C_{T,treat,ref}$ is the threshold cycle number of the reference gene in the same group as outlined in the multiple regression model described by Yuan *et al*. (41). A negative $2^{-\Delta\Delta C_T}$ value suggests that a gene in the DMBA-only treated group (group

3) was over expressed compared to the same gene in the untreated control group (group 6). This analysis was performed for each target gene and a *p*-value was obtained for each gene. A simultaneous test was performed using Bonferroni's method for multiple comparisons of the nine target genes. The overall significance level for multiple comparisons was 0.05. For a single target gene, an expression level with a *p*-value smaller than 0.005 was considered to be significantly different. The same analysis was applied to differences in gene expression levels between the DMBA- and LS-treated (group 5) and normal cheek pouches (group 6). Subsequently, comparisons of gene expression levels were made between DMBA plus LS (group 5) and DMBA-treated (group 3) cheek pouches.

## Results

*Animal food consumption and body weights*. In the CCB, there were no significant differences in the mean of food consumed in the three groups over the 12-week period. The average consumption per hamster was 6.292 and 6.124 g/day for animals on 10% and 5% LS and 6.134 g/day for AIN-76A controls. In the PCB, the mean food consumption for animals on the 10% LS diet for six weeks was 6.986 g/day/hamster, which was significantly higher than that of hamsters on control diet ($p<0.01$). The mean food consumption of animals on 5% LS was 6.563 g/day/hamster, higher than that of the control diet group, but not significantly different ($p=0.086$).

There were no significant differences in body weight between the three groups (10%, 5% LS and control diet) in the CCB. At the time of harvest, the average weights of animals receiving 10% and 5% LS diets were 127.33±21.85 and 131.33±20.13 g in contrast to 133.83±14.69 g for hamsters on the control diet. In the PCB, the mean body weight of animals on the 10% LS diet was significantly higher ($p<0.01$) than that of the 5% LS and control diet groups at harvest. Average weights were 149.57±14.58, 138±18.88, and 133.83±14.69 g, respectively, for animals on 10% LS, 5% LS and control diets.

*Inhibition of lesions and tumors by LS. CCB:* The chemopreventive effect of LS on total lesions and tumors in hamster cheek pouch is shown in Table II. There were 133 and 134 total lesions in cheek pouches of 21 hamsters given 10% or 5% LS pellets in contrast to 174 in 20 surviving animals given control pellets ($p<0.01$). Among the 133 total lesions in animals treated with 10% LS, there were 58 tumors, 63 leukoplakias, and 12 papillomas, whereas among the 134 lesions in hamsters treated with 5% LS, there were 71 tumors, 52 leukoplakic lesions, and 11 papillomas. Out of the total lesions in 20 animals on control pellets, there were 94 tumors, 67 leukoplakias and 13 papillomas.

The reduction in total tumors in the CCB was due to a decrease in the number of tumors per cheek pouch in hamsters fed 5% LS ($p=0.04$) or 10% LS ($p<0.01$) rather than a reduction in the number of animals with tumors. In hamsters fed 10% LS, only five out of the 42 cheek pouches (11.9%) had three or more tumors compared with 11 out of 42 pouches of the 5% LS group (26.2%) and 16 out of 40 (40%) cheek pouches in the control group ($p=0.03$). Tumor incidence was 95%, 90.5%, and 100% for 10% and 5% LS and DMBA controls, respectively, leading to the conclusion that the reduction in the total number of tumors was due to a decrease in tumors per animal. Tumors ranged in size ($Vmm^3=W^2\times L\times 0.52$) from 1.04 $mm^3$ to 1,348 $mm^3$; there were no significant differences in individual tumor sizes between the three groups at the time of sacrifice.

*PCB:* In order to develop an oral cancer model that would mimic conditions found in human oral mucosa after being exposed to exogenous carcinogens, the HCPs were first treated with carcinogens for six weeks followed by six weeks of LS pellet administration. When harvested at 12 weeks, the inhibition of total lesions and tumors was equal to (or exceeded) the degree of inhibition observed when LS pellets were given in the CCB (Table II). With 10% LS in the diet, there were 127 total lesions (63 tumors, 59 leukoplakias, and five papillomas) and with 5% LS there were 122 lesions (46 tumors, 70 leukoplakias, and six papillomas) compared to the 174 total lesions in the controls ($p<0.01$). As found in the CCB, the reduction in tumors was due to a decrease in tumors per animal in both treatment groups ($p<0.01$). In the 10% LS group, there were nine out of 42 cheek pouches with 3 or more tumors (21.4%) and in the 5% LS-treated animals there were six out of 42 pouches (14.3%) with three or more tumors in contrast to 16 out of 40 pouches in the DMBA control group (40%). Similar to the outcome in the CCB, individual tumor sizes were not significantly different between the three groups.

*Molecular analyses*. rRT-PCR analyses were performed on HCPs harvested at 12 weeks (with tumors removed) from hamsters treated with DMBA alone (group 3) and with DMBA plus 5% LS post-initiation (group 5) and each compared to cheek pouches from untreated controls (group 6). There were 12 cheek pouches analyzed from nine hamsters in the DMBA control group, and 16 cheek pouches from 11 hamsters in the DMBA plus 5% LS-treated group. rRT-PCR was run in triplicate using 10 TaqMan Gene Expression assays (nine target genes and one endogenous reference gene) for each cheek pouch. *Actb* was used as the endogenous reference gene for analysis based on data from Livak and Schimittgen (40). These comparisons revealed a significant increase ($p<0.001$) in expression of $p13^{Arf}$, *p16,* and *p53* (tumor-suppressor genes) and *Bcl2* (anti-apoptosis gene) in HCPs of hamsters (after tumor removal) when treated only with DMBA for six weeks and fed control diet for the six week experimental period (Figure 1). After DMBA treatment of cheek pouches for six weeks, followed by six weeks with 5% LS pellets, there was a significant reduction ($p<0.05$) in expression of *p16*

Table II. *Percentage inhibition of DMBA-induced oral lesions by strawberries.*

| Group, Treatment[a] | N | Lesions/M[b] | Tumors/M[c] | Leukoplakias/M[d] | Papillomas/M[e] |
|---|---|---|---|---|---|
| 1,10% Strawberries | 21 | 133/6.33* | 58/2.76* | 63/3.00 | 12/0.57 |
| Complete Assay | | 27.3%[f] | 41.3% | 10.5% | 12.4% |
| 2, 5% Strawberries | 21 | 134/6.38* | 71/3.38** | 52/2.48 | 11/0.52 |
| Complete Assay | | 26.7% | 28.1% | 26% | 20% |
| 3,10% Strawberries | 21 | 127/6.05* | 63/3.00* | 59/2.81 | 5/0.23 |
| Post-initiation | | 30.5% | 36.2% | 16.2% | 64.7% |
| 4, 5% Strawberries | 21 | 121/5.76* | 46/2.19* | 70/3.33 | 6/0.29 |
| Post-initiation | | 33.8% | 53.5% | 1.0% | 54.4% |
| 5, DMBA Controls | 20[g] | 174/8.7 | 94/4.70 | 67/3.35 | 13/0.65 |
| No Strawberries | | 0 | 0 | 0 | 0 |
| 6, Sentinel Animals no DMBA or Berries | 11 | 0 | 0 | 0 | 0 |

[a]Hamster cheek pouches were painted with DMBA and LS pellets dispensed as described in Table I. N, number of animals per group. [b]Lesions/M. Lesions=tumors, leukoplakias, papillomas. M=No. of lesions/hamster. [c]Tumors/M. Total number of tumors >1 mm/total per hamster. [d]Leukoplakias/M. Leukoplakias were defined as small (<1 mm in diameter), white, flat lesions/total per hamster. [e]Papillomas/M. Papillomas appeared as exophytic growths with dense keratinized tissue atop thin (~0.5 mm) finger-like fronds/total per hamster. [f]Percentage inhibition based on DMBA controls. [g]One animal died one week prior to sacrifice in the DMBA control group. Significantly different from DMBA controls at *$p \leq 0.01$; **$p = 0.04$.

and *p13$^{Arf}$* and a significant increase in expression of *p53* and *Bcl2* when compared to DMBA treatment only. Expression of Cyclin B2 mRNA [a G(2)-M cyclin, that binds to CDK2 and CDC2 kinases and the cell-cycle regulators, pRb, E2F-1, and p21] was nonsignificantly increased by DMBA treatment alone and was reduced only slightly after LS treatment. Expression of *Bax, Cdk2, c-Myc*, and *Vegf* was not significantly altered in DMBA and LS-treated cheek pouches when compared to DMBA treatment only.

*Histological analysis*. After tumor removal, sections from both cheek pouches from hamsters treated with 10% and 5% LS pellets and pouches from DMBA control hamsters were H&E stained and examined microscopically for histological lesions (mild dysplasia, severe dysplasia, and CIS). Mild dysplasia was characterized by changes in the epithelium such as basilar crowding and hyperplasia, cellular disorganization, and maturational disturbances not extending more than one-third of the epithelial thickness with little interruption of the keratin layer. Severe dysplasia included the above parameters extending beyond one-half of the epithelial thickness but not affecting the entirety of the epithelium. Additional features included frequent mitotic figures, cellular pleomorphism, nuclear atypia, and some early disturbance of the keratin layer. CIS appeared as a full thickness epithelial change with the above features, an expansion of multiple layers of cells into the suprabasal and intermediate layers, and with disturbance of the keratin layer but without penetration of the basement membrane. The results of the microscopic examination are presented in Table III and Figure 2. There were very significant decreases ($p<0.001$) in the occurrence of mild and severe dysplasia in cheek pouches from hamsters continuously treated (CCB) with 10% LS and of mild dysplasia in cheek pouches treated with 5% LS ($p=0.002$), whereas the numbers of CIS increased with continuous 5% LS treatment ($p<0.01$). When treated post-initiation (PCB) with 10% or 5% LS, there was no decrease in the number of pre-existing dysplasias in contrast to the reduction in tumors. A number of residual tumors were observed in some of the histological sections of cheek pouches, representing the balance of tumor tissue that remained after resection of tumors at time of harvest. Control tissues, from hamsters treated only with DMSO, had a normal histological appearance with a normal orthokeratin pattern and no evidence of a hyperproliferative or inflammatory response.

## Discussion

The prevalence is likely to rise for decades based upon the expanding global exposure to tobacco products. Despite major advances in detection and treatment over the past 30 years, the overall survival rates have not appreciably changed and remain discouraging for oral cancer that has spread beyond the mucosa. Thus, primary prevention by coupling tobacco and alcohol cessation with diet/chemoprevention is of vital importance. In addition, this disease provides an opportunity for secondary prevention, defined as the prevention of recurrent or new secondary primary oral malignancies. Indeed, 20% of patients with oral SCC will exhibit recurrence within 18 months post-surgery and 22-42% will present with a second primary tumor within 5-8 years (6, 7, 8). The critical question remains: Which approaches for primary and secondary prevention will prove most effective and safe for those at high risk for this disease?

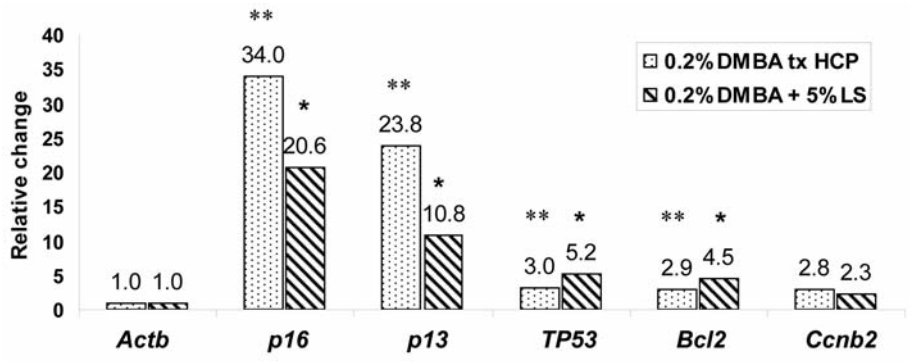


Figure 1. *Changes in gene expression in dysplastic hamster cheek pouchs (HCP) after six weeks of post-initiation treatment with 5% lyophilized strawberries (LS). Analyses were performed on cheek pouches from hamsters treated with 7,12-dimethylbenz(a)anthracene (DMBA) alone, DMBA plus 5% LS post-initiation, and cheek pouches from untreated controls. Real-time reverse transcription polymerase chain reactions (RT-PCR) were run in triplicate using 10 TaqMan Gene Expression assays (nine target genes and one endogenous reference gene) for each cheek pouch with Actb as the endogenous reference gene. There was a significant increase (**p<0.001) in expression of p13, p16, and p53 (tumor-suppressor genes) and B-cell CLL/lymphoma 2 (Bcl2, anti-apoptosis gene) in cheek pouches of hamsters treated with DMBA when contrasted to untreated control HCP. After treatment of cheek pouches for six weeks with 5% LS pellets, there was a significant reduction in expression of p16 and p13 and a significant increase in expression of p53 and Bcl2 (*p<0.05) when compared to DMBA treatment only. Cyclin B2 (Ccnb2) RNA expression was nonsignificantly increased by DMBA treatment and was reduced slightly in expression after LS treatment.*

Major risk factors for oral cancer in the United States are use of tobacco and alcoholic beverages, which account for ~75% of all oral cancers (10). Other risk factors include malnutrition and poor dietary intake of essential minerals, and may also include exposure to viruses such as human papillomavirus (42, 43) and Epstein-Barr virus (43). In addition, there are data to suggest that marijuana use may increase the risk for oral cancer (44). The International Report on Cancer Prevention from the American Institute for Cancer Research estimates that 30% of all cancers can be prevented by not using tobacco and another 30% to 40% can be avoided by attention to the foods we eat, proper exercise, and weight (45).

Epidemiological studies have shown a strong correlation between consumption of fresh fruits and vegetables and a decreased oral cancer risk (11). In support of these observations, research teams at The Ohio State University have pursued a series of pre-clinical investigations demonstrating the chemopreventive activity of lyophilized black raspberries and LS and have identified potential mechanisms of action. Current experimental data in hamsters suggest that LS are as effective as lyophilized black raspberries in inhibiting development of oral cancer. Although the data are highly promising, the potential chemopreventive activity of such preparations in human oral cancer has not yet been thoroughly evaluated.

Numerous chemopreventive agents have been shown to inhibit development of tumors in the HCP when administered before, during, or after initiation by chemical carcinogens (33). These chemopreventive agents possess several mechanisms of action including: de-toxification of metabolites, stimulation of phase II enzymes, protease inhibition, scavenging of reactive oxygen, inhibition of cell proliferation, inhibition of angiogenesis, stimulation of p53, and inhibition of DNA adduct formation (33).

Natural and synthetic compounds are especially effective for chemoprevention of oral cancer in HCP. In addition to our findings with LS, β-carotene (46), retinyl acetate (47), *Ocimum sanctum* (48), diallyl sulfide (49), protease inhibitors from soybeans (50), green tea polyphenols (51), and several other synthetic compounds have been shown to prevent oral tumor formation when given after initiation by carcinogens.

The anomalous data that show a greater inhibition of tumors in HCP by 5% *versus* 10% LS in the PCB are similar to those found for esophageal tumors in rats by 5% and 10% black raspberries and strawberries. In these previously published experiments, Kresty *et al.* (13) demonstrated that diets containing lyophilized black raspberries fed to rats for two weeks prior to carcinogen and for 30 weeks during carcinogen treatment (CCB), yielded a tumor multiplicity that decreased from 3.15 tumors per control animal to 1.93 and 1.61 with 5% and 10% lyophilized black raspberries, respectively. However in a PCB, inhibition by 5% lyophilized black raspberries was greater at week 25 than that observed for 10% as multiplicity decreased from 1.40 in controls to 0.53 and 0.80, respectively, and by week 35, only 5% demonstrated a significant inhibitory activity. A similar pattern was described by Carlton *et al.* (15), who observed 24.4% and 56.1% inhibition of esophageal tumors by 5% and 10% LS in a CCB and 37.5% and 31.3% by 5% and 10% berries in a post-initiation experiment. Casto *et al.* (12) also found that 5% of lyophilized black raspberries were more inhibitory than 10% for oral tumors in the HCP, and Harris *et al.* (14) reported that at lower concentrations they yielded a greater inhibition of aberrant crypt foci in rat

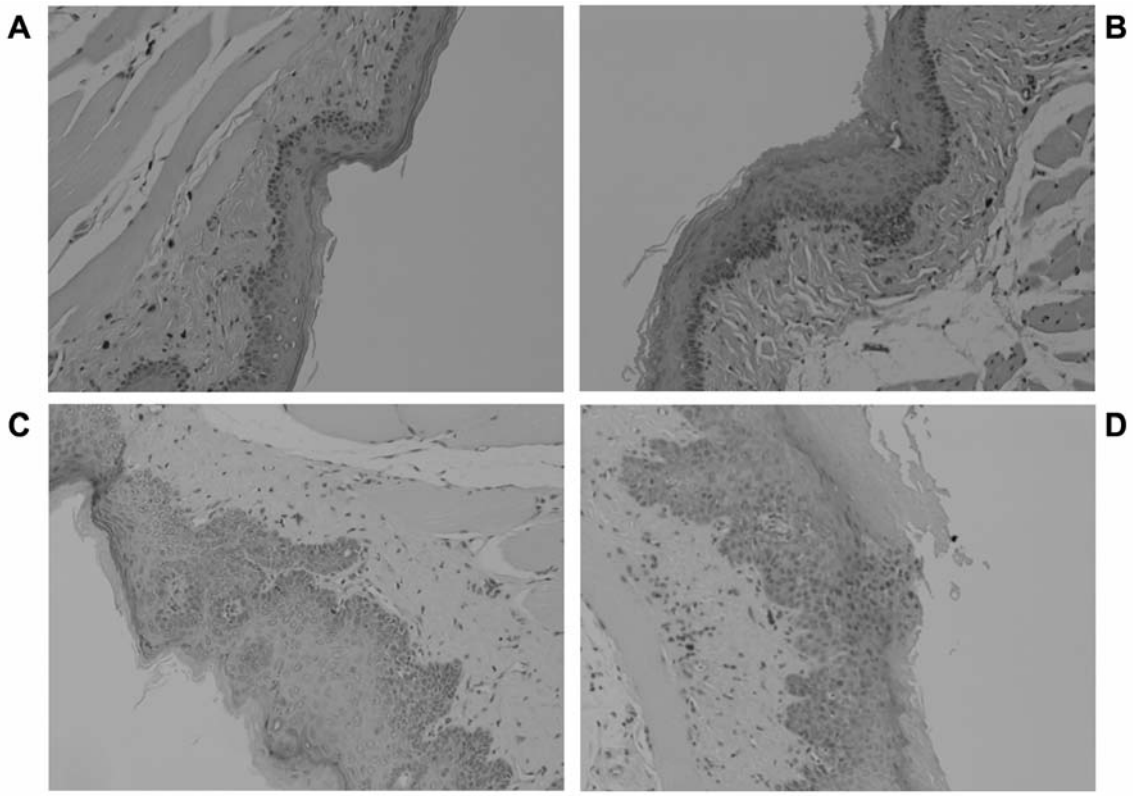


Figure 2. *Hematoxylin and eosin-stained sections of hamster cheek pouch 12 weeks after commencement of DMBA treatment. A: Normal epithelium, B: mild dysplasia with adjacent area of hyperplasia, C: severe dysplasia, and D: carcinoma-in-situ (×200).*

Table III. *Summary of histology of hamster cheek pouches (HCPs) treated with 10% and 5% lyophilized strawberries (LS) for 12 weeks.*

| Treatment[a] DMBA + LS | Mild dysplasia[b] /multiplicity | Severe dysplasia[b] /multiplicity | Carcinoma *in situ*[b] /multiplicity | Total lesions[c] /multiplicity |
|---|---|---|---|---|
| CCB | | | | |
| 10% LS (N=41) | 449/10.95[d] | 150/3.66[d] | 34/0.83 | 633/15.44 |
| 5% LS (N=41) | 555/13.54[e] | 266/6.49 | 64/2.05[f] | 885/21.59 |
| DMBA Control (N=40) | 648/16.20 | 227/5.67 | 39/0.97 | 914/22.85 |

[a]Hamsters were treated as described in Table I. N, Number of pouches evaluated. [b]Properties of mild and severe dysplasias and carcinoma *in situ* are defined in the Results section. [c]Total of mild and severe dysplasias and carcinoma *in situ*. [d,e]Significantly inhibited when compared to DMBA controls ($p<0.001$ and $p=0.002$, respectively). [f]Significantly increased when compared to DMBA controls ($p<0.01$).

colon, as opposed to higher concentrations. The increased inhibition by 10% berries over 5% berries in the CCB may be due in part to the inhibition of initiating events, primarily the formation of DNA adducts. Casto *et al*. (12), Kresty *et al*. (13), and Carlton *et al*. (15) demonstrated a reduction in DNA adducts when berries were given prior to and during carcinogen treatment. It has been speculated that failure to show a dose response with berries may be due to their complex composition (13). Not only do they contain powerful anti-oxidants and other tumor-inhibitory substances, but also contain an abundance of micronutrients, phenolics, phytosterols, carotenoids, iron, and potentially competing components that may act as promoting agents or interfere with inhibitory pathways as the concentration of berry powder in the diet increases from 5% to 10% or above. In addition, by increasing the concentration of berries in food, it is conjectured that the polyphenolics contained therein may become pro-oxidant, leading to an enhancement of carcinogenesis; such a mechanism has been suggested by Lee and Lee (52) and Galati and O'Brien (53).

The histopathological analysis of cheek pouches with removed tumors that were treated with 5% and 10% LS reveals that the LS are very effective in reducing the number of early lesions when given prior to, during, and after carcinogen treatment; the multiplicity of both mild and severe dysplasia were reduced to levels compatible with the reduction in number of tumors. As further evidence for this apparent effect on early lesions, when the tumor sizes (1-10, 10-100, and >100 $mm^3$) were plotted against the number of tumors, it was revealed that the greatest reduction in numbers of tumors (when compared to controls) was in the groups of smaller and mid-sized tumors (data not shown). However, there was little or no reduction in the size of large tumors, indicating that the maximum effect of LS may have been manifested on early-developing tumors. There was no reduction of pre-existing dysplastic lesions when LS were added after carcinogen treatment, strongly suggesting that LS inhibited progression to malignancy of both pre-existing and newly-arising dysplasia. This resulted in an accumulation of pre-malignant lesions concurrent with the reduction in numbers of tumors. A similar situation has been observed in mouse lung chemoprevention studies by Pereira *et al*. (54) and Gunning *et al*. (55, 56), in which histopathological examination showed that the chemopreventive agents inhibited progression to carcinomas resulting in an accumulation of adenomas and papillary adenomas in the carcinogen-initiated lungs, concomitant with a reduction in lung carcinomas.

Gene mRNA expression in tumor-denuded cheek pouches from hamsters treated with DMBA alone and DMBA with 5% LS post-initiation (which yielded the greatest reduction in number of tumors) were contrasted to expression in cheek pouches from untreated hamsters. These comparisons revealed a significant increase ($p<0.001$) in expression of $p13^{Arf}$ [GenBank Accession AF443796, which has a 76% to 80% identity to human $p14^{Arf}$ and rat and mouse $p19^{Arf}$ nucleotide sequences (T. Knobloch, unpublished data)], *p16*, and *p53* (tumor-suppressor genes), and *Bcl2* (anti-apoptosis) in cheek pouches of hamsters treated with DMBA. After six weeks of feeding with 5% LS pellets, there was a significant reduction ($p<0.05$) in expression of *p16* and $p13^{Arf}$ and a significant increase in expression of *p53* and *Bcl2* when compared to DMBA treatment only. Overexpression of *p16* was reported by Weinberger *et al*. (57) to result in a decreased recurrence rate and increased survival rate in patients with oropharyngeal tumors. Lang *et al*. showed that 55% of tumors from the head and neck had *p16* over-expressed; primarily resulting from point-mutations and intragenic deletions (58). In our current studies, the frequency of *p16* alterations was 70.6% in DMBA-induced tumors, the majority (67.6%) of which were deletions or methylation (59), that is consistent with the incidence found in human oral SCC.

It has been shown that tumors and dysplastic tissues that are positive for HPV frequently overexpress *p16* (60, 61, 62). The presence of a hamster papilloma virus has been described in dysplastic and tumor tissues from Syrian Golden hamsters following DMBA application (63). In the current study, when DMBA-induced hamster cheek pouch dysplasia and tumors were examined for the hamster oral papilloma virus E6 and E7 genes (GenBank Submission E15111), expression was found in five out of five samples with a higher response in dysplastic tissues than in the corresponding SCC tumor (Warner, unpublished data). These data suggest that the overexpression of *p16* may have been associated with the presence of the hamster papilloma virus.

Expression of *Bax, Cdk2, c-Myc,* and *Vegf* were not significantly altered in cheek pouches from hamsters treated with DMBA plus LS when compared to hamsters treated with DMBA only. It should be noted that the cheek pouches examined for changes in gene expression were devoid of tumors and were taken six weeks after cessation of carcinogen treatment. Experiments are currently underway to evaluate the ability of short-term treatment with LS to modulate gene expression when given for 14 days, beginning 48 h after the last carcinogen treatment.

In summary, demonstration of tumor inhibition by concurrent and post-initiation administration in hamsters, and the inhibitory effects on progression of early dysplasia, advances the feasibility of using strawberries for preventive and mechanistic studies in human oral cancer; such studies have been performed by Chen *et al*. in human patients with esophageal dysplasia (64). The data presented herein show, to our knowledge for the first time, that incorporation of LS in the diet can inhibit oral tumor formation in an experimental model. Furthermore, administration of LS after induction of moderate-to-severe dysplasia was just as effective in inhibiting tumor formation as administration of LS before, during, and after carcinogen treatment. The various chemoprevention assays in the HCP provide an opportunity to evaluate the effects of chemopreventive agents in a well-defined animal system that mimics the pathological condition in former tobacco users.

## Acknowledgements

These studies were supported by an Independent Contractor Agreement with the California Strawberry Commission and with funds provided by the Molecular Carcinogenesis and Chemoprevention Program, The Ohio State University Comprehensive Cancer Center. We wish to thank Dr. Gary D. Stoner, former Director of the Cancer Chemoprevention and Support Program, The Ohio State University, for his input and support of our investigations into chemoprevention of oral cancer by berries.

The Authors declare that they have no conflicts of interest associated with this study and have no competing financial interests

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*Received September 24, 2013*
*Revised October 21, 2013*
*Accepted October 23, 2013*