The Chemopreventive Effects of Tea on Human Oral Precancerous Mucosa Lesions (44369)

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Abstract. **A double-blind intervention trial was conducted in patients with oral mucosa leukoplakia using a mixed tea product developed by the authors. Fifty-nine oral mucosa leukoplakia patients, diagnosed by established clinical and pathological criteria, were randomly divided into a treated group (3 g mixed tea oral administration and topical treatment) and a control group (placebo and glycerin treatment). After the 6-month trial, the size of oral lesion was decreased in 37.9% of the 29 treated patients and increased in 3.4%; whereas the oral lesion was decreased in 10.0% of the 30 control patients and increased in 6.7%. At the same time, the incidence of micronucleated exfoliated oral mucosa cells in the treated group (5.4 per 1000 cells) was lower than that in the control group (11.3 per 1000 cells)($P < 0.01$); whereas it was 1.4 per 1000 cells in 20 healthy subjects. The micronuclei and chromosome aberration rate in the peripheral blood lymphocytes showed the same results. In pathological examination, there were significant differences ($P < 0.05$) in the number and total volume of the silver-stained Nucleolar Organizer Regions (AgNOR) and the proliferating index of Proliferation Cell Nuclear Antigen (PCNA) in oral mucosa cell nuclei between the treated group and the control group which indicates that cell proliferation was decreased in the treated patients. The overall results provide some direct evidence on the protective effects of tea on oral cancer.** [P.S.E.B.M. 1999, Vol 220]

Tea is one the most popular beverages consumed worldwide. Many laboratory studies have demonstrated that tea has antimutagenic and anticarcinogenic effects in various laboratory testing systems and animal models using different carcinogens (1–3). However, whether tea has a preventive effect on human cancer is an unsolved issue. Although several epidemiological studies suggested a protective effect of tea consumption on certain types of human cancer, other studies have indicated an opposite effect, and no clear-cut conclusions could be drawn (4, 5). The inconsistency may be attributed to some confounding factors, such as very hot tea, tobacco, and alcohol (6). It is widely agreed that to conduct intervention trials of tea on human cancer is an important approach to elucidate protective effects of tea.

Oral cancer is the sixth commonest cancer throughout the world, particularly in some developing countries, such as India, Sri Lanka, Vietnam, Philippines, and parts of Brazil, where up to 25% of all cancers are oral cancer (7, 8). In recent decades, oral cancer incidence and mortality rates have been increasing in the United States, Japan, Germany, and China (9). Oral leukplakia is a well established precancerous lesion of oral cancer, and people with oral leukoplakias are at high risk for oral cancer (10). In general, 2%–12% of patients with oral leukoplakia lesions will eventually develop malignant oral cancer and for the nonhomogeneous type of lesions, chances of developing oral cancer are as high as 15%–40% (11). Therefore, oral leukoplakia has been used as an ideal model for studying the effects of chemopreventive agents in the prevention of oral cancer. A number of antioxidant micronutrients have been showed to be effective in clinical trials on oral leukoplakia (12, 13).

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In our previous short-term screening tests on tea ingredients, we found that the effects of any single tea ingredient (tea polyphenols, tea catechins, tea pigments, tea polysaccharide, etc.) on the initiation, promotion, or progression phase of carcinogenesis were not as strong as the whole tea water extracts (14). Based on this concept, a mixed tea product was developed in our laboratory in which tea polyphenols and tea pigments were added to the whole water extracts of tea in a proportion based on short-term screening tests. Our previous studies showed that this mixed tea had stronger protective effects than the whole water extract or tea polyphenols against precancerous lesions in rat liver induced by N-nitrosodiethylamine (15). In another recent animal study on oral cancer, we found that the drinking of both green tea and this mixed tea reduced the incidence of oral cancer induced by 7,12-dimethylbenz(a)anthracene in Syrian golden hamsters by 42.6% and 67.2%, respectively. This paper reports our findings in a randomized, placebo-controlled, double-blind chemopreventive trial of the mixed tea on human oral leukoplakia. The purpose is to find out whether the mixed tea treatment could reduce the chances of developing human oral cancer from precancerous lesions.

Materials and Methods

Subjects and Treatments. Sixty-four cases of oral leukoplakias, 40 males and 24 females between 23 and 28 years of age and diagnosed by oral pathological examination were chosen from the Beijing Dental Hospital and volunteered for the intervention trial. Before treatment, all the subjects underwent a complete oral examination and the number, size, and type of lesions were carefully recorded. A simple questionnaire was administered to record their habits of smoking, alcohol-drinking, and tea-drinking. Among them, 46 subjects were smokers, and the mean smoking pack-years (packs/day/person × smoking years) was 19.5 years. The subjects were randomly divided equally into the tea-treated and placebo group, with 32 subjects in each group. During the 6-month clinical trial, 29 subjects in the tea-treated group and 30 subjects in the placebo group completed the trial. Twenty nonsmoking dental patients without oral leukoplakia matched for age and sex were chosen from the Beijing Dental Hospital as the healthy control group.

The mixed tea was provided by the Institute of Tea Science and Research, Chinese Academy of Agricultural Science, and was composed of a dried mixture of the whole water extract of green tea, green tea polyphenols (40%), and tea pigments in the ratio of 4:1:1. Tea pigments are the oxidized product of 40% green tea polyphenols and are composed primarily of theaflavins, thearubigins, and theabrownin. The mixed tea was put into capsules; each capsule contained 0.38 g of mixed tea. Subjects in the tea-treated group took eight capsules (3 g mixed tea, 2 capsules q.i.d.) and at the same time painted (t.i.d.) on the lesions topically with mixed tea in glycerin at the concentration of 10%. Subjects in the placebo group received the same amount of starch capsules and painted the same amount of starch containing glycerin as the tea-treated group. During the trial, subjects were asked not to take any special medicines, including vitamins and to avoid exposure to X-rays. The amount of vegetables and fruits consumed each day was recorded.

Clinical Examination. The subjects were examined every 2 months during the trial by a dentist who did not know in which group each subject was. The size and number of lesions of each subject at the baseline and at the end of the trial were recorded. A complete regression was defined as the complete disappearance of the lesions. A partial regression was defined as 30% or more reduction in the size of a single lesion or in the sum of sizes of multiple lesions. Lesions with no change in size were recorded as no change. Deterioration referred to the occurrence of new lesions. The differences between the tea-treated and placebo group were analyzed by the X^2-test.

Biomarkers of DNA Damage. *Micronuclei in exfoliated oral mucosa cells.* Exfoliated cells were obtained by scraping the buccal mucosa from sites with and without lesions by a moistened wooden tongue depressor at baseline and the end of 3 months and 6 months of the trials. Exfoliated cells from normal mucosa of the healthy control subjects were also obtained. Each mucosa scraping was smeared onto two microscope slides, which were air-dried and fixed in methanol/glacial acetic acid (3:1). The slides were stained with Feuglen staining and counterstained with Fast Green (16). Two thousands cells were counted blindly, and the number of micronucleated cells per 1000 cells was reported.

Micronuclei and chromosome aberration in peripheral blood lymphocytes. Intravenous blood was collected from each leukoplakia patients at the beginning and the end of the trial and from healthy controls at the beginning of the trial. Then 0.3 ml whole blood was added to 5 ml RPMI 1640 culture medium (Gibco Company, Grand Island, NY) containing 10% fetal serum, 100 unit/ml penicillin solution, 100 μg/ml streptomycin solution, and 0.01 unit/ml phytohemagglutinin. After incubating at 37°C for 72 hr, the lymphocytes were treated with hypotonic KCI (0.053 mol) for 1 min and fixed in methanol/acetic acid (3:1) for two times. The lymphocytes were spread on two slides and stained with 10% Giemsa for 10 min. One thousands lymphocytes were examined blindly, and the number of micronucleated cells per 1000 lymphocytes was referred as the incidence of micronucleated cells.

Another 0.3 ml whole blood was added to 5 ml RPMI 1640 culture medium, and incubated at 37°C for 66 hr. Colchicine (Sigma Company, St. Louis, MO) (final concentration 20 μg/ml) was added to each culture, and 1.5 hr later, the lymphocytes were treated with hypotonic KCI (0.053 mol) for 20 min and fixed in methanol/acetic acid (3:1) for three times. The lymphocytes were spread on three slides and stained with 10% Giemsa for 10 min. One hundreds diploids division metaphase were scored for all classified aberrations, including chromosome breaks and acentoic and

acentric rings. The number of chromosome aberrations per 100 cells was referred as the rate of chromosome aberration.

Biomarkers of Cell Proliferation. Oral mucosa biopsies were conducted at the beginning and the end of the trial from similar sites. Tissues were fixed in 10% formalin, dehydrated, and embedded in paraffin. Several sections were cut at 4 μm and mounted on polysine-coated glass slides, and the deparaffinized sections were then stained with hemotoxylin and eosin for routine histopathological examination and also for silver stained Nucleolar Organizer Regions (AgNOR), Proliferation Cell Nuclear Antigen (PCNA), and Epidermal Growth Factor Receptor (EGFR) analysis. Differences both between the tea-treated and placebo group as well as before and after the trial were compared by the Student *t* test.

The AgNOR technique was performed according to Ploton *et al.* (17). Briefly, the colloid silver solution for staining the NORs was prepared by dissolving gelatin in 1% aqueous formic acid at a concentration of 2%. This solution was mixed with 60% aqueous silver nitrate as the working solution. The slides were incubated with the colloid solution for 60 min at room temperature, protected from sunlight, then washed with deionized water. Morphometric analysis was perfomed with a MPIAS-500 image analyzer and processing system, then 100 cells per slide were examined. The number of AgNOR dots per nucleus and the volume of single AgNOR dots (VNOR) and the total volume of AgNOR dots (TVNOR) were calculated automatically. The values of volume were expressed in μm^2.

PCNA staining was performed using the Avidin-Biotin Peroxidase Complex immunoperoxidase method (18). The slides were emersed in 0.01 mol citrate buffer at 95°C for 10 min to recover antigen. The activity of endogenous peroxidases was blocked by methanol containing 3% H_2O_2 for 10 min. Then the slides were treated with normal sheep serum to reduce nonspecific reactions and incubated at 4°C overnight with anti-PCNA monoclonal antibody (Vector Company) dilution in 1:50. Slides were then reacted with biotinylated anti-mouse secondary antibody and Avidin-Biotin Peroxidase Complex (purchased from Vector company) for 30 min at 37°C. The reactivity was carried out with 0.03% diaminobenzidine and H_2O_2. The slides were counterstained with hematoxylin. The slides without primary monoclonal antibody treatment served as the negative control. All cells with stained nuclei were considered positive cells. Ten random fields were counted using a 40× objective. The proliferating index of PCNA was expressed as the number of cells with positive staining per 100 cells counted.

The EGFR staining was the same as the PCNA staining method, but antigen recovery was used by adding 0.1% trypsin and incubating at 37°C for 30 min and the primary antibody was anti-EGFR monoclonal antibody (Vector Laboratories, Inc., Burlingame, CA)(dilution in 1:100). When stained nuclear membrane was found, the cell was identified as the positive cell. Ten random fields were counted using a 40× objective. The results were expressed as the number of positive EGFR expression cells per 100 cells counted.

Results

The general features of subjects (Table I) show that there were no significant differences in age, sex, smoking, alcohol-drinking, and tea-drinking behavior as well as clinical and pathological types of leukoplakia patients between the tea-treated and placebo groups. No significant differences in the amount of vegetables and fruits consumed during the trial between the two groups were observed (Table II). All the subjects continued with their usual lifestyle, including smoking, tea-drinking and alcohol-drinking during the trial period, and no special medicines were taken, except that 11% of the subjects used some antibiotics and anticold drugs. No X-ray exposure was recorded.

Clinical Manifestations. After 6 months of tea intervention, partial regression of the lesions was observed in 11 of the 29 (37.9%) cases, no change in 17 (58.6%) cases, and deterioration in 1 (3.4%) case. In the placebo group, partial regression of the lesions was found in 3 of the 30 (10.0%) cases, no change in 25 (83.3%) cases and deterioration in 2 (6.7%) cases. The partial regression rate was significantly higher in the tea-treated group than in the placebo group ($P < 0.05$) (Table III).

Biomarkers of DNA Damage. The data in Table IV show that frequency of micronucleated exfoliated buccal cells in both lesion sites and normal sites of the mucosa of leukoplakia patients was higher than in healthy subjects ($P < 0.01$). In the same oral leukoplakia subjects, the frequency of micronucleated cells was higher in the mucosa cells from the lesion sites than in those from the normal sites ($P < 0.01$). After 3 months and 6 months of tea treatment, micronuclei formation in the cells from both the lesion sites and normal sites decreased significantly ($P < 0.01$) in the tea-treated group, whereas no significant changes were found in the placebo group.

The frequency of micronucleated cells and chromo-

Table I. General Characteristics of Subjects

	Tea-treated (n = 29)	Placebo controls (n = 30)	Healthy controls (n = 20)
Age (years)	53.7	55.4	51.5
Males:females	18:11	17:13	10:10
Smokers	24	22	0
Alcohol-drinkers	6	5	0
Tea-drinkers	7	9	4
Homogeneous leukoplakia	24	24	0
Nonhomogeneous leukoplakia	5	6	0
Hyperplastic leukoplakia	22	25	0
Dysplastic leukoplakia	7	5	0

Table II. Vegetable and Fruit Consumption in Leukoplakia Patients During the Trial (g/person/day)

	Tea-treated (n = 29)[a]	Placebo controls (n = 30)[a]
Vegetables[b]	317 ± 115	323 ± 109
Fruits[b]	39.6 ± 41.0	35.1 ± 23.7

[a] Figures in parentheses are number of subjects.
[b] Vegetable and fruit consumption between the two groups were not statistically different by t test, $P > 0.05$.

Table III. Changes of Clinical Manifestations in Leukoplakia Patients After 6-Month Tea Treatment

	Tea-treated (n = 29)[a]	Placebo controls (n = 30)[a]
Partial regression[b]	11 (37.9%)	3 (10.0%)
No change	17 (58.6%)	25 (83.3%)
Deterioration	1 (3.4%)	2 (6.7%)

[a] Figures in parentheses are number of subjects.
[b] The rate of partial regression was significantly different between the two groups by X^2-test, $P < 0.05$.

some aberration in peripheral blood lymphocytes of leukoplakia patients was significantly higher than in the healthy subjects (Tables V, VI). After 6 months of tea intervention, the frequency of micronucleated cells and chromosome aberration decreased significantly ($P < 0.01$) in the tea-treated group, whereas no significant changes were found in the placebo group.

Biomarkers of Cell Proliferation. After 6 months of tea treatment, the number of AgNOR dots per nucleus, TVNOR, and the proliferating index of PCNA decreased significantly ($P < 0.01$) in the tea-treated group, whereas no significant changes were found in the placebo group (Tables VII, VIII, IX). No changes in VNOR were observed in both the tea-treated and placebo group.

The percentage of EGFR positive cells was reduced after 6 months of tea treatment; however, it was not statistically significant ($P > 0.05$) (Table X).

Discussion

Intervention studies in human population using cancer incidence as an end point require large numbers of subjects, long-term follow-up, and a large budget, which have severely limited the number of chemopreventive studies. The use of intermediate biomarkers to serve as surrogate end points for cancer incidence in chemopreventive trials has attracted the attention of most scientists (19–21). In the current intervention trial, we used several biomarkers as intermediate end points to assess the protective effects of tea on human oral cancer in oral leukoplakia patients, including clinical manifestations and biomarkers of DNA damage and cell proliferation in oral mucosa.

From the data reported above, after 6 months of trials, 37.9% of the subjects in the tea-treated group showed a reduction in the size of leukoplakia lesions, whereas only 10.0% of subjects in the placebo group showed a reduction. The results indicate that tea treatment can improve the clinical manifestations of the oral lesions.

DNA damage is considered a crucial mechanism in cancer development (22). Micronuclei formation reflects the extent of ongoing DNA damage and has been shown to be correlated with cancer risk at several sites, such as oral, esophagus, lung, and bladder, and therefore was often used as a biomarker of early carcinogenesis (23–25). Micronuclei of oral buccal mucosa cells are formed in the basal cells and migrate to the epithelial surface and are detected in exfoliated cells. It was reported that cigarette smoking caused an increase in micronuclei counts in exfoliated oral buccal mucosa cells in smokers, and it was thought to reflect the increased oral cancer risk (16). The frequency of micronucleated exfoliated buccal cells in oral leukoplakias was higher than in cells from normal mucosa sites, especially in smokers (26). Several previously reported intervention trials in a high-risk population for oral cancer supported the use of micronuclei frequency in exfoliated buccal cells as an intermediate biomarker (27, 28) because the specimen are easy to obtain noninvasively and micronuclei could be measured quantitatively and repeatedly. On the other hand, peripheral blood lymphocytes have been used extensively in biomonitoring of populations exposed to various mutagenic or carcinogenic compounds. Using DNA damage in peripheral blood lymphocytes as a biomarker is based on the hypothesis that the extent of genetic damage in peripheral blood lymphocytes reflects similar events in the precursor cells for carcinogenic processes in the target tissues. An increased frequency of micronuclei and chromosome aberration in peripheral blood lymphocytes has been considered indicative of increased cancer risk (29). Dreosti (30) suggested the use of micronuclei and chromosome aberration in peripheral blood lymphocytes as economical and valid biomarkers in intervention trials.

In our current study, the frequency of micronucleated exfoliated buccal cells and the frequency of micronucleated cells and chromosome aberration in peripheral blood lymphocytes in the leukoplakia patients were significantly higher than in healthy subjects. This is probably due to the fact that 80% of the subjects were smokers, and the average pack-years were about 20 years. After 6 months of mixed tea intervention, the micronuclei formation in exfoliated oral cells was reduced from 10.5 to 5.4 per 1000 cells, and the micronucleated cells and chromosome aberration rate in the peripheral blood lymphocytes were reduced from 3.9 to 2.6 per 1000 cells and from 2.5 to 1.7 per 100 cells, respectively. These results show that tea has a significant chemopreventive effect on DNA damage.

Abnormal cellular proliferation is another important mechanism in carcinogenesis. Indicators of proliferation may be used as intermediate biomarker for chemoprevention research. Nucleolar organizer regions (NOR) are loops of DNA that contain ribosomal RNA genes. These genes are transcribed by RNA polymerase I and ultimately direct ri-

Table IV. The Number of Micronucleated Exfoliated Buccal Cells in Leukoplakia Patients per 1000 Cells at Baseline, 3 Months and 6 Months of Trial

	Tea-treated (n = 29)		Placebo controls (n = 30)		Healthy controls (n = 20)
	Lesion	Normal mucosa	Lesion	Normal mucosa	
Baseline	10.50 ± 5.29[a,b]	5.20 ± 2.79[a]	10.10 ± 4.07[a,b]	5.12 ± 2.04[a]	1.40 ± 0.61
3-month	6.68 ± 3.21[c,d]	3.89 ± 1.86[e]	10.35 ± 4.07	4.82 ± 2.53	
6-month	5.39 ± 3.05[c,d]	3.05 ± 1.62[c,d]	11.30 ± 4.29	5.46 ± 2.90	

Note. All values are mean ± SD. Figures in parentheses are numbers of subjects.
[a] Significantly different from healthy controls by Possion test, $P < 0.01$.
[b] Significantly different from normal mucosa by Possion test, $P < 0.01$.
[c] Significantly different from baseline by Possion test, $P < 0.01$.
[d] Significantly different from placebo group by Possion test, $P < 0.01$.
[e] Significantly different from baseline by Possion test, $P < 0.05$.

Table V. The Number of Micronucleated Cells per 1000 Peripheral Blood Lymphocytes in Leukoplakia Patients Before and After Trial

	Tea-treated (n = 29)	Placebo controls (n = 30)	Healthy controls (n = 20)
Baseline	3.89 ± 1.47[a]	4.00 ± 1.46[a]	0.9 ± 0.71
6-month	2.62 ± 1.32[b,c]	4.36 ± 1.94	

Note. All values are mean ± SD. Figures in parentheses are numbers of subjects.
[a] Significantly different from healthy controls by Possion test, $P < 0.01$.
[b] Significantly different from baseline by Possion test, $P < 0.01$.
[c] Significantly different from placebo controls by Possion test, $P < 0.01$.

Table VI. Chromosome Aberration Rate in Peripheral Blood Lymphocytes in Leukoplakia Patients Before and After Trial (%)

	Tea-treated (n = 29)	Placebo controls (n = 30)	Healthy controls (n = 20)
Baseline	2.48 ± 1.29[a]	2.26 ± 1.31	0.70 ± 0.59
6-month	1.69 ± 1.03[b,c]	2.60 ± 1.42	

Note. All values are mean ± SD. Figures in parentheses are numbers of subjects.
[a] Significantly different from healthy controls by Possion test, $P < 0.01$.
[b] Significantly different from baseline by Possion test, $P < 0.05$.
[c] Significantly different from placebo controls by Possion test, $P < 0.01$.

Table VII. The Number of AgNOR Dots per Nucleus in Leukoplakia Lesions of Oral Mucosa Before and After Trial

	Tea-treated (n = 22)	Placebo controls (n = 21)
Baseline	6.34 ± 2.19	6.24 ± 2.01
6-month	4.44 ± 3.80[a,b]	6.10 ± 2.71

Note. All values are mean ± SD. Figures in parentheses are number of subjects.
[a] Significantly different from baseline by t test, $P < 0.01$.
[b] Significantly different from placebo controls by t test, $P < 0.05$.

bosome formation and protein synthesis. NOR-associated acidic proteins related to sites of r-RNA transcription can be demonstrated in the interphase nucleus in histologic sections by silver-staining, which permits precise localization of NOR sites. These silver reaction products can be visualized as black dots, which are referred to as AgNORs. It has been suggested that the number and size of AgNOR dots in a nucleus may reflect the status of cell activation and therefore reflect proliferation (31). PCNA is a helper protein of the DNA δ-polymerase , reaching an expression peak during the S-phase of the cellular cycle and playing an important role in cellular proliferation (32). Several studies have demonstrated that AgNOR and PCNA are both increased during the progression from normal epithelium to hyperplasia and dysplasia and ultimately carcinoma *in situ* and may be related with degree of the dysplasia and malignancy of lesions examined (33, 34). Therefore, AgNOR and PCNA are potential cell proliferation biomarkers for carcinogenesis.

In our chemopreventive trial, after 6 months of tea intervention, the numbers of AgNORs/per nucleus and TVNOR, as well as the proliferating index of PCNA in the tea-treated group were significantly decreased, whereas there were no significant changes in the placebo group. These results show that mixed tea treatment have a significant inhibitory effect on oral mucosa cell proliferation.

EGFR is a product of the *erb* oncogene, and the overexpression of EGFR has been involved in the pathogenesis of certain epithelial neoplasms (35). In this study, the rate of EGFR expression in the tea-treated group decreased after 6 months of treatment and was also lower than that of the placebo group, but the differences were not statistically significant ($P > 0.05$). This may be due to the large individual variation of EGFR expression rates and relatively small sample size (Table X).

The results from various intermediate markers in this clinical trial indicate that the mixed tea is able to improve the precancerous changes in oral leukoplakia patients by protecting DNA damage and inhibiting proliferation of oral mucosa cells, and suggest that the mixed tea might have certain preventive effects in human oral cancer. This is in line with our animal studies that showed a protective role of tea on DMBA-induced oral carcinogenesis in Syrian ham-

Table VIII. The Volume of a Single AgNOR Dot and Total AgNOR Dots per Nucleus in Leukoplakia Lesions of Oral Mucosa Before and After Trial

	Tea-treated (n = 22)		Placebo control (n = 21)	
	VNOR	TVNOR	VNOR	TVNOR
Baseline	1.87 ± 0.71	16.3 ± 2.80	1.61 ± 0.45	13.94 ± 4.41
6-month	2.00 ± 1.72	11.78 ± 2.71[a]	1.91 ± 0.91	14.37 ± 5.10

Note. All values are mean ± SD. Figures in parentheses are numbers of subjects.
[a] Significantly different from baseline by t test, $P < 0.01$.

Table IX. Proliferating Index of PCNA in Leukoplakia Lesions of Oral Mucosa Before and After Trial

	Tea-treated (n = 22)	Placebo controls (n = 21)
Baseline	36.2 ± 22.9	37.3 ± 22.8
6-month	24.3 ± 16.5[a,b]	39.0 ± 23.4

Note. All values are mean ± SD. Figures in parentheses are numbers of subjects.
[a] Significantly different from baseline by t test, $P < 0.05$.
[b] Significantly different from placebo controls by t test, $P < 0.05$.

Table X. Percentage of EGFR Positive Cells in Leukoplakia Lesions of Oral Mucosa Before and After Trial

	Tea-treated (n = 22)	Placebo controls (n = 21)
Baseline	36.4 ± 25.8	35.8 ± 26.5
6-month	32.2 ± 20.4[a]	36.7 ± 26.5

Note. All values are mean ± SD. Figures in parentheses are numbers of subjects.
[a] Comparisons between baseline and 6-month values of tea-treated group, as well as between tea-treated group and placebo group are not statistically significant by t test, $P > 0.05$.

sters by reducing the tumor formation at the oral buccal pouch, preventing DNA damage, and inhibiting cell proliferation (36). The clinical improvements associated with positive impact on DNA damage and cell proliferation, both in animals and human studies, suggest that these biomarkers used in conjunction with precancerous lesions can serve as valuable intermediate end points in chemoprevention studies.

Smoking is the best recognized risk factor for oral precancerous lesions and oral cancer. Abundant free radicals in cigarette smoke are believed to initiate tobacco-related cancers (including oral cancer) by damaging DNA (37). It was reported that the level of antioxidant nutrients in serum of leukoplakia patients was lower than in healthy subjects (38). Therefore, the protective mechanism of tea on oral cancer may be attributed to its antioxidant capacity by quenching highly reactive single oxygen and free radical species.

In conclusion, although the sample size and the time of treatment of this intervention trial were limited, the results from this study have provided some encouraging and direct evidence on the preventive effects of tea on human cancer.

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1. Mukhtar H, Wang ZH, Katiyar SK, Agareal R. Tea Components: Antimutagenic and anticarcinogenic effects. Prev Med **21:**351–360, 1992.
2. Yang CS, Wang ZY. Tea and cancer. J Natl Cancer Inst **85:**1038–1049, 1993.
3. Wang ZY, Cheng JS, Zhou ZC. Antimutagenic activity of green tea polyphenols. Mutat Res **223:**273–285, 1989.
4. Katiyar SK, Mukhtar H. Tea in chemoprevention of cancer: Epidemiological and experimental studies (review). Int J Oncol **8:**221–238, 1996.
5. Kohlmeier L, Weterings KGC, Steck S, Kok FJ. Tea and prevention: An evaluation of the epidemiological literature. Nutr Res **27:**1–13, 1997.
6. Victora CG, Munoz N, Hong JGE. Hot beverages and esophageal cancer in southern Brazil: A case-control study. Int J Cancer **39:**710–716, 1987.
7. Parkin SM, Laara E, Muir CS. Estimates of the worldwide frequency of sixteen major cancers. Int J Cancer **41:**184–197, 1988.
8. Magrath I, Litvakk J. Cancer in developing countries: Opportunity and challenge. J Natl Cancer Inst **85:**862–874, 1993.
9. Tanaka T. Chemoprevention of oral carcinogenesis. Oral Oncol, Eur J Cancer **31B:**3–15, 1995.
10. Vecchia CL, Tavani A, Franceschi S, Levi F, Corrao G, Negri E. Epidemiology and prevention of oral cancer. Oral Oncol **33:**302–312, 1997.
11. Sankaranarayanan R, Mathew B, Varghese C, Sudhakaran PR, Menon V, Jayadeep A, Nair MK, Mathews C, Mahalingam TR, Balaram P, Nair PP. Chemoprevention of oral leukoplakia with vitamin A and β-carotene: An assessment. Oral Oncol **33:**231–236, 1995.
12. Kaugars GE, Silverman S, Ma Lovas JGL Jr., Thompson JS, Brandt RB. Use of antioxidant supplements in the treatment of human oral leukoplakia. Oral Surg Oral Med Oral Pathol **81:**13, 1996.
13. Prasad MPR, Mukundan MA, Krishnaswamy K. Micronuclei and carcinogen DNA adducts as intermediate end points in nutrient intervention trial of precancerous lesions in the oral cavity. Oral Oncol, Eur J Cancer **31B:**155–159, 1995.
14. Liu LJ, Han C, Chen JS. Short-term screening of anticarcinogenic ingredients of tea by cell biology assays. J Hyg Res **27:**53–56, 1998.
15. Qi L, Han C. The inhibitory effects of tea and tea-mixture on diethylnitrosamine-induced precancerous hepatic lesions in rats and its antioxidative mechanisms. J Hyg Res **26:**382–386, 1997.
16. Stich HF, Rosin MP. Quantitating the synergistic effect of smoke and alcohol consumption with the micronucleus test on human buccal mucosa cells. Int J Cancer **31:**305–308, 1983.
17. Ploton D, Menager M, Jeannesson P. Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. Histochem J **18:**5–14, 1986.

18. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroixdase complex (ABC) in immunoperoxidase techniques: A comparision between ABA qbd unlabelled PAP procedures. J Histochem Cytochem **29:**577–580, 1981.
19. Greenwald P, Witkin KM, Malone WF. The study of markers of biological effect in cancer prevention research trials. Int J Cancer **52:**189–196, 1992.
20. Boone CW, Kelloff GJ, Steele VE. The natural history of intraepithelial neoplasia: Relevance to the search for intermediate end point biomarkers. J Cell Biochem (Suppl 16G):23–26, 1992.
21. Lippman SM, Lee JS, Lotan R. Biomarkers as intermediate end points in chemoprevention trials. J Natl Inst Cancer **82:**554–560, 1992.
22. Weinstein IB. The origins of human cancer: Molecular mechanisms and their implications for cancer prevention and treatment. Cancer Res **48:**4135–4143, 1988.
23. Poppel GV, Kok FJ, Hermus RJJ. β-carotene supplementation in smokers reduces the frequency of micronuclei in sputum. Br J Cancer **66:**1164–1168, 1992.
24. Reali DF, Dimarino S, Bahramandpour A, Carducci R. Micronuclei in exfoliated urothelial cells and urine mutagenicity in smokers. Mutat Res **192:**145–149, 1987.
25. Garewal HS, Ramsey L, Kaugars G, Boyle J. Clinical experience with the micronucleus assay. J Cell Biochem (Suppl **17F):**206–212, 1993.
26. Desai SS, Ghaisas SD, Jakhi SD, Bhide SV. Cytogenetic damage in exfoliated oral mucosal cells and circulating lymphocytes of patients suffering from precancerous oral lesions. Cancer Lett **109:**9–14, 1996.
27. Stich HF, Stich W, Rosin MP, Vallejera MO. Use of the micronucleus test to monitor the effect of vitamin A, β-carotene and canthaxanthin on the buccal mucosa of betel nut/tobacco chewers. Int J Cancer **34:**745–750, 1984.
28. Benner SE, Lippman SM, Wargovich MJ. Micronuclei, a biomarker for chemoprevention trials: Results of a randomized study in oral premalignancy. Int J Cancer **59:**457, 1994.
29. Hagmar L, Brogger A, Hansteen IL, Heim S. Cancer risk in human predicted by increased levels of chromosomal aberrations in lymphocytes: Nordic study group on the health risk of chromosome damage. Cancer Res **54:**2919–2922, 1994.
30. Dreosti IE. Cancer biomarkers in the field of tea. Cancer Lett **114:**319–321, 1997.
31. Fakan S, Hernadez-Verdun D. The nucleus and the nucleolar organizer regions. Biol Cell **56:**186–206, 1986.
32. Hall PA, Levinson DA, Woods AL. Proliferating cell nuclear antigen (PCNA) inmmunolocalization in paraffin sections: An index of cell proliferation with evidence of deregulated expression in some neoplasm. J Pathol **162:**285–294, 1990.
33. Muzio LL, Mignogna MD, Staibano S, Vico GD. Morphometric study of nucleolar organizer regions (AgNOR) in HPV-associated precancerous lesions and microinvasive carcinoma of the oral cavity. Oral Oncol **33:**247–259, 1997.
34. Storkel S, Reichert T, Reiffen KA. EGFR and PCNA expression in oral squamous cell carcinomas: A valuable tool in estimating the patient's prognosis. Oral Oncol, Eur J Cancer **29B:**273–277, 1993.
35. Shin DM, Ro JY, Hong WK. Dysregulation of epidermal growth factor receptor expression in premalignant lesions during head and neck tumorigenesis. Cancer Res **54:**3153–3159, 1994.
36. Li N, Han C, Chen JS. The efficacy of various tea profucts on DMBA-induced oral carcinogenesis in hamsters. J Hyg Res **28:**1999.
37. Pryou WA. Cigarette smoke and the involvement of free radical reactions in chemical carcinogenesis. Br J Cancer **55** (Suppl VIII): 19, 1987.
38. Ramaswamy G, Rao VR, Kumaraswamy SV, Anantha N. Serum vitamins' status in oral leukoplakias: A preliminary study. Oral Oncol, Eur J Cancer **32B:**120–122, 1996.