Archives of Oral Biology 70 (2016) 130–142

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/aob

Chemopreventive effect of *Toona sinensis* leaf extract on 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch squamous cell carcinogenesis

Wen-Chen Wang[a,b,c,d], Ching-Yi Chen[a,c,1], Hseng-Kuang Hsu[e], Li-Min Lin[a,b,c], Yuk-Kwan Chen[a,b,c,1,*]

[a] Division of Oral Pathology and Maxillofacial Radiology, Department of Dentistry, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[b] School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[c] Oral and Maxillofacial Imaging Center, Kaohsiung Medical University, Kaohsiung, Taiwan
[d] Department of Dentistry, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung, Taiwan
[e] Department of Physiology, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

ARTICLE INFO

Article history:
Received 30 April 2015
Received in revised form 10 June 2016
Accepted 12 June 2016

Keywords:
Toona sinensis
DMBA-carcinogenesis
Hamster
Chemoprevention

ABSTRACT

Objective: Toona sinensis leaf extract (TSL) has been shown to have anti-tumor effects on cancer cell lines. This study aimed to investigate the chemopreventive potential and the underlying mechanism of TSL during 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis.
Methods: One hundred hamsters were divided into control (n = 30), carcinogenic (n = 20), preventive (n = 42), and therapeutic (n = 8) groups. The animals in carcinogenic and preventive groups were administered reverse osmosis water (carcinogenic group) or TSL (1 g/kg bw) (preventive group) by gavage daily for 4 weeks, and their bilateral pouches were painted with a 0.5% DMBA solution for 4, 9, and 12 weeks. The animals in the therapeutic group were treated with DMBA for 12 weeks prior to TSL administration for 4 weeks. Expression levels of survivin, X chromosome-linked inhibitor of apoptosis (XIAP), proliferating cell nuclear antigen (PCNA), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) proteins were analyzed by immunohistochemistry. Apoptotic activity was examined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method, cytochrome C, and poly (ADP-ribose) polymerase (PARP).
Results: In the preventive group, the results showed significant decreases not only in the incidences of squamous cell carcinoma (SCC) (50%) and epithelial dysplasia (62.5%) but also in the tumor number, tumor volume, tumor burden, and the severity of dysplastic lesions. The down-regulation of survivin, XIAP, PCNA, iNOS, and COX-2 proteins and the increased apoptotic activity indicated anti-proliferative and apoptosis-inducing abilities of TSL on DMBA-induced HBP carcinogenesis.
Conclusions: The results suggested that TSL might be a promising candidate for the prevention of oral cancer.

1. Introduction

Oral cancer is the eleventh most common cancer in the world, with the highest incidences occurring in South and Southeast Asia (e.g., Sri Lanka, India, and Taiwan) (Ferlay et al., 2013). Despite advances in treatment (surgical techniques, chemotherapy, and radiation treatment) for oral cancer, the prognosis has not improved significantly over the past few decades. The five-year survival rate for oral cancer remains at approximately 50% (Brinkman & Wong, 2006; Warnakulasuriya, 2009). Additionally, despite efforts in public education and screening, the proportion of patients at an advanced stage of the disease has not changed in the past 40 years (McGurk, Chan, Jones, O'Reagan, & Sherriff, 2005; Warnakulasuriya, 2009) with the occurrence of a second primary tumor to be in 10–35% of cases (van Oijen & Slootweg, 2000).

* Corresponding author at: School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan.
E-mail address: k0285@ms22.hinet.net (Y.-K. Chen).
[1] These two authors contributed equally to this paper.

http://dx.doi.org/10.1016/j.archoralbio.2016.06.015
0003-9969/

Therefore, early detection and prevention are believed to be more effective in improving survival by decreasing the incidence and disease severity of oral cancer.

Cancer chemoprevention is defined as the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression (Sporn, Dunlog, Newton, & Smith, 1976). A large number of chemopreventive agents that have been developed from dietary agents, natural plants, and synthetic chemicals, have been examined, including retinoids (Hong et al., 1986), tea polyphenols (Chandra Mohan, Devaraj, Prathiba, Hara, & Nagini, 2006), black raspberries extract (Han, Ding, Casto, Stoner, & D'Ambrosio, 2005), curcumin (Manoharan, Balakrishnan, Menon, Alias, & Reena, 2009), limonoids (Harish Kumar, Vidya Priyadarsini, Vinothini, Vidjaya Letchoumy, & Nagini, 2010), COX-2 inhibitor (Nishimura et al., 2004), EGFR inhibitor (Sun et al., 2008), etc. Only a few agents have been tested in small clinical trials. The results have supported the efficacy of their chemopreventive potential, but disadvantages such as high toxicity, prominent adverse effects, and quick recurrence have also been observed.

Toona sinensis is an edible medicinal plant. TSL extract has been shown to have an anticancer effect on several cancer cell lines *in vitro* (Chang et al., 2006; Chen et al., 2009; Hseu et al., 2011; Wang et al., 2010; Yang et al., 2006; Yang et al., 2010), including oral cancer (Chia, Rajbanshi, Calhoun, & Chiu, 2010). The main component of TSL extract responsible for the cancer inhibition was identified to be gallic acid, which can induce apoptosis via reactive oxygen species (Chen et al., 2009; Chia et al., 2010) without significant toxicity to normal human oral keratinocytes (Chia et al., 2010). It was also reported that TSL extract can arrest the cell cycle and induce apoptosis by inhibiting the expression of Bcl2 in human lung cancer cells (Wang et al., 2010; Yang et al., 2010). In addition, TSL extract was found to be effective in terms of inhibiting proliferation and inducing apoptosis in a human oral cancer cell line by increasing the expression of TNF-α and reducing the expressions of survivin and cIAP (Chia et al., 2010). However, the influence of TSL extract on premalignant lesions and its effect on oral cancer in animal models have not been examined in the published English literature. Therefore, the current study aimed to evaluate the effects of TSL extract on malignant and premalignant lesions in a 7,12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch carcinogenesis model. The mechanism was explored by analyzing the immunohistochemical expressions of PCNA, survivin, XIAP, iNOS, COX-2, cytochrome C, and poly(ADP-ribose) polymerase (PARP), as well as the results of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays.

2. Materials and methods

2.1. Chemicals

DMBA was purchased from Sigma Chemical Company (St. Louis, MO, USA). TSL extract was purchased from Taiwan *Toona Sinensis* Biotechnical Corporation (Kaohsiung, Taiwan). The leaves were obtained from *Toona sinensis* Roem grown in Tuku (Yunlin County, Taiwan) and were picked and washed thoroughly with water. Reverse osmosis (RO) water was added to the leaves in the proportion of 4 L of RO water to 1 kg of leaves. The mixture was heated and maintained at boiling point for 30 min and then cooled slowly for 2 h at room temperature. The leaves were then removed, and the remaining fluid was filtered using a sieve (70-mesh). The filtered concentrate was lyophilized using Virtis apparatus to obtain a crude extract. Using this procedure, 100 g of leaves yielded approximately 5–6 g of lyophilized TSL powder.

2.2. Animals and diets

One-hundred male Syrian golden hamsters (*Mesocricatus auratus*), aged 6–7 weeks and weighing approximately 100 g each, were supplied from the National Science Council Animal Breeding Center (Taipei, Taiwan). The animals were housed under constant conditions (22 °C, 12-h light/dark cycle) and fed with tap water and standard Purina laboratory chow *ad libitum*. Appropriate animal care and an approved experimental protocol ensured humane treatment, and all procedures were approved by the Kaohsiung Medical University Bioethics Committee and were conducted in accordance with the NIH Guide for the Care and Use of Animals.

2.3. Treatment protocol

The animals were randomly divided into four major groups: carcinogenic group (groups 1–3), preventive group (groups 4–9), therapeutic group (group 10), and control group (groups 11–13). The number of animals in each group and their treatments are listed in Table 1. After allowing the animals one week of acclimatization to their new surroundings, all hamsters in the carcinogenic group were given RO water by gavage five times per week for 4 weeks (Supplementary Fig. S1). Both buccal pouches of the hamsters in the carcinogenic group were sedated three times per week by means of a mixture of ketamine and xylazine (50%), and then painted with a 0.5% solution of DMBA in mineral oil, using a number 4 sable-hair brush, for 4 (group 1), 9 (group 2), and 12 (group 3) weeks. The animals in the preventive group underwent TSL extract administration (0.1 g TSL1 powder mixed with 1 mL RO water) by gavage at a concentration of 1 g/kg body weight for 4 weeks. In groups 4–6, the animals were then treated with DMBA painting only for 4 (group 4), 9 (group 5), and 12 (group 6) weeks. Subsequent to TSL administration for 4 weeks, the animals in groups 7–9 underwent both TSL extract administration by gavage and DMBA painting for 4 (group 7), 9 (group 8), and 12 (group 9) weeks. They were administered TSL extract first by gavage, and then DMBA painting was applied 30 min later. In the therapeutic group, DMBA painting for 12 weeks was administered first, followed by TSL extract administration by gavage for 4 weeks. The animals in group 11 underwent TSL extract administration by gavage for 4 weeks, and no further treatment followed. In group 12, the hamster pouches were painted with mineral oil for 12 weeks. Group 13 served as an untreated control. The animals in groups 1, 4,

Table 1
Animal number, treatment protocol, weight, tumor number, tumor volume, and tumor burden of each group (mean ± SD).

Group	Number	Treatment protocol
Carcinogenic groups		
1	5	RO water 4 weeks + DMBA 4 weeks
2	5	RO water 4 weeks + DMBA 9 weeks
3	10	RO water 4 weeks + DMBA 12 weeks
Preventive groups		
4	5	TSL 4 weeks + DMBA 4 weeks
5	5	TSL 4 weeks + DMBA 9 weeks
6	14	TSL 4 weeks + DMBA 12 weeks
7	5	TSL 4 weeks + DMBA & TSL 4 weeks
8	5	TSL 4 weeks + DMBA & TSL 9 weeks
9	8	TSL 4 weeks + DMBA & TSL 12 weeks
Therapeutic group		
10	8	DMBA 12 weeks + TSL 4 weeks
Control groups		
11	10	TSL 4 weeks
12	10	Mineral oil 12 weeks
13	10	No treatment

TSL: *Toona sinensis* leaves extract; RO: reverse osmosis water.

and 7 were sacrificed in the 8th week; the animals in groups 2, 5, and 8 were sacrificed in the 13th week; the animals in groups 3, 6, 9, 10, and 11 were sacrificed in the 16th week; and the animals in groups 12 and 13 were sacrificed in the 12th week.

2.4. Specimen collection

The animals were simultaneously sacrificed by CO_2 inhalation (3 days after the last treatment) at 9 AM to avoid any influence of diurnal variation (Lin & Chen, 1997). The number of tumor growths was counted, and the diameter of tumors was measured. The entire pouches were then serially sectioned and routinely processed for light microscopy by fixing in 10% neutral-buffered formalin solution for approximately 24 h, dehydrating in a series of ascending concentration alcohol solutions, cleaning in xylene, and embedding in paraffin for hematoxylin-eosin staining and immunohistochemical study.

2.5. Immunohistochemistry

The antibodies used in the present study were as follows: rabbit polyclonal antibodies against human, rat, and mouse for survivin, XIAP, PCNA, iNOS, COX-2, cytochrome C (Cat. Nos. ab469, ab21278, ab2426, ab15323, ab 15191, ab 76237 Abcam Corporation, Cambridge, UK), and PARP (Cat. No. G7341 Promega Corporation, WI, USA), and their specificity has been established in previous studies (Baldasquin-Caceres, Gomez-Garcia, López-Jornet, Castillo-Sanchez, & Vicente-Ortega, 2014; Chen & Lin, 2000; Hsue, Chen, & Lin, 2008; Jiang & Wang, 2004; Kockx, Muhring, Knaapen, & de Meyer, 1998; Nishimura et al., 2004). The tissue sections were deparafinized in xylene 3 times and rehydrated in a graded series of ethanol (absolute, 95%, 70%) and distilled water. In order to retrieve antigenicity, the sections were soaked in citrate buffer (10 mM; pH 6.0) and placed in a pressure cooker at 121 °C for 6 min. The sections were allowed to cool and then washed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked with 3% H_2O_2 in distilled water for 5 min. Non-specific antibody binding was inhibited by incubating the sections in a 10% solution of normal rabbit serum for 1 h. The sections were subsequently washed in PBS and then incubated with a 1:100 dilution of primary antibodies against survivin, XIAP, PCNA, iNOS, COX-2, cytochrome C, and PARP overnight at 4 °C. A biotin-streptavidin detection system was used (Dako, Santa Barbara, CA, USA). After being rinsed with PBS, the sections were incubated with the linking reagent (biotinylated anti-immunoglobulins) for 20 min at room temperature. Following rinsing with PBS, the sections were later incubated in a peroxidase-conjugated streptavidin label solution for 10 min at room temperature. After being rinsed with PBS again, the sections were incubated in deaminobenzidine (DAB, Roche, Cat. No. 1718096) and then counterstained with hematoxylin. Each set of experiments included a human buccal SCC specimen known to express survivin, XIAP, PCNA, iNOS, COX-2, and PARP, which served as a positive control and ensured the reproducibility of the staining process. A negative control, in which the primary antibody step was omitted, was also included in each set of experiments.

2.6. TUNEL assay

TUNEL assay was performed according to the protocol described by the manufacturer of the Roche *in situ* cell death detection kit, POD (Cat. No. 11684817910). The tissue sections were deparaffinized as described above and then washed in PBS for 5 min. The sections were soaked with protein kinase K working solution (20 mg/ml) for 15–30 min at room temperature. Following washing with PBS 3 times (5 min each time), TUNEL reaction mixture was added to the sections. The sections were subsequently covered with a lid and incubated for 1 h at 37 °C in a humidified atmosphere in the dark. After being rinsed with PBS 3 times, converter-POD (1:1 dilution) solution was added to the sections. The sections were incubated for 30 min at 37 °C and subsequently rinsed with PBS 3 times. After being incubated in DAB, the sections were counterstained with hematoxylin, dehydrated, cleaned in xylene, and mounted.

2.7. Evaluation methods

The number of visible tumors on each pouch was counted. The length, width, and height of each tumor were measured in millimeters, and the tumor volume was calculated using the formula $4/3\ \pi$ (length/2)(width/2)(height/2). The tumor burden of each pouch was determined by adding all tumor volumes of the pouch.

Histopathological analysis, including cell counting of the staining of PCNA, survivin, XIAP, iNOS, cytochrome C, and PARP

Table 2
– Body weight, tumor number, tumor volume, and tumor burden of each group (mean ± SD).

Group	Body weight (g)	Tumor number**	Tumor volume* (mm^3)	Tumor burden** (mm^3)
Carcinogenic groups				
1	104.46 ± 6.49	0 ± 0	0 ± 0	0 ± 0
2	118.48 ± 5.87	0 ± 0	0 ± 0	0 ± 0
3	99.23 ± 10.85	7.22 ± 4.52	24.97 ± 27.44	222.64 ± 273.63
Preventive groups				
4	121.95 ± 9.95	0 ± 0	0 ± 0	0 ± 0
5	116.55 ± 17.30	0 ± 0	0 ± 0	0± 0
6	120.20 ± 8.46	0.75 ± 0.93	10.12 ± 28.88	10.65 ± 28.79
7	116.85 ± 9.56	0 ± 0	0 ± 0	0 ± 0
8	107.61 ± 11.11	0 ± 0	0 ± 0	0 ± 0
9	114.11 ± 6.24	0.69 ± 0.79	6.74 ± 18.99	7.82 ± 19.18
Therapeutic group				
10	104.06 ± 11.17	5.06 ± 2.49	68.33 ± 99.48	273.94 ± 265.63
Control groups				
11	110.32 ± 6.44	0 ± 0	0 ± 0	0 ± 0
12	101.27 ± 5.26	0 ± 0	0 ± 0	0 ± 0
13	96.19 ± 8.21	0 ± 0	0 ± 0	0 ± 0

TSL: *Toona sinensis* leaves extract; RO: reverse osmosis water.
* Significant differences between groups 3, 6, 9, and 10 by ANOVA followed by the Tukey-Kramer method ($P < 0.05$).
** Significant differences between groups 3, 6, 9, and 10 by ANOVA followed by the Tukey-Kramer method ($P < 0.0001$).

Table 3
Histopathological diagnoses, incidence of SCC and incidence of dysplasia in each group.

Group	Histopathological diagnoses (%)								Incidence (%)	
	N	HK	EH	MiED	MoED	SED	CIS	SCC	Dysplasia	SCC
1	–	–	20	80	–	–	–	–	80	0
2	–	–	–	–	40	60	–	–	100	0
3	–	–	–	–	–	–	–	100	0	100
4	–	10	30	60	–	–	–	–	60	0
5[d]	–	–	28.57	28.57	28.57	14.29	–	–	71.43	0
6[e,f]	–	–	–	–	21.43	25	–	53.57	46.43	53.57[b,c]
7	20	–	–	60	–	20	–	–	80	0
8[d]	–	25	37.5	25	12.5	–	–	–	37.5 [a]	0
9[e]f	–	–	–	–	25	18.75	6.25**	50	50	50[b,c]
10	–	–	–	–	–	–	–	100	0	100
13	100								0	0

N: Normal; HK: hyperkeratosis; EH: epithelial hyperplasia; MiED: mild epithelial dysplasia; MoED: moderate epithelial dysplasia; SED: severe epithelial dysplasia; CIS: carcinoma *in situ*; SCC: squamous cell carcinoma.
[a] Significantly different from group 2 by the χ^2 test ($P < 0.05$).
[b] Significantly different from group 3 by the χ^2 test ($P < 0.05$).
[c] Significantly different from group 10 by the χ^2 test ($P < 0.05$).
[d] Significant differences from group 2 in histopathological diagnoses.
[e] Significant differences from group 3 in histopathological diagnoses.
[f] Significant differences from group 10 in histopathological diagnoses.

and TUNEL assays of each pouch was performed under light microscopy independently by two experienced pathologists. If consensus between the two pathologists could not be reached, a third pathologist was requested to make the final decision.

The results of histopathological observation were classified into the following categories: normal (N), hyperkeratosis (HK), epithelial hyperplasia (EH), mild epithelial dysplasia (MiED), moderate epithelial dysplasia (MoED), severe epithelial dysplasia

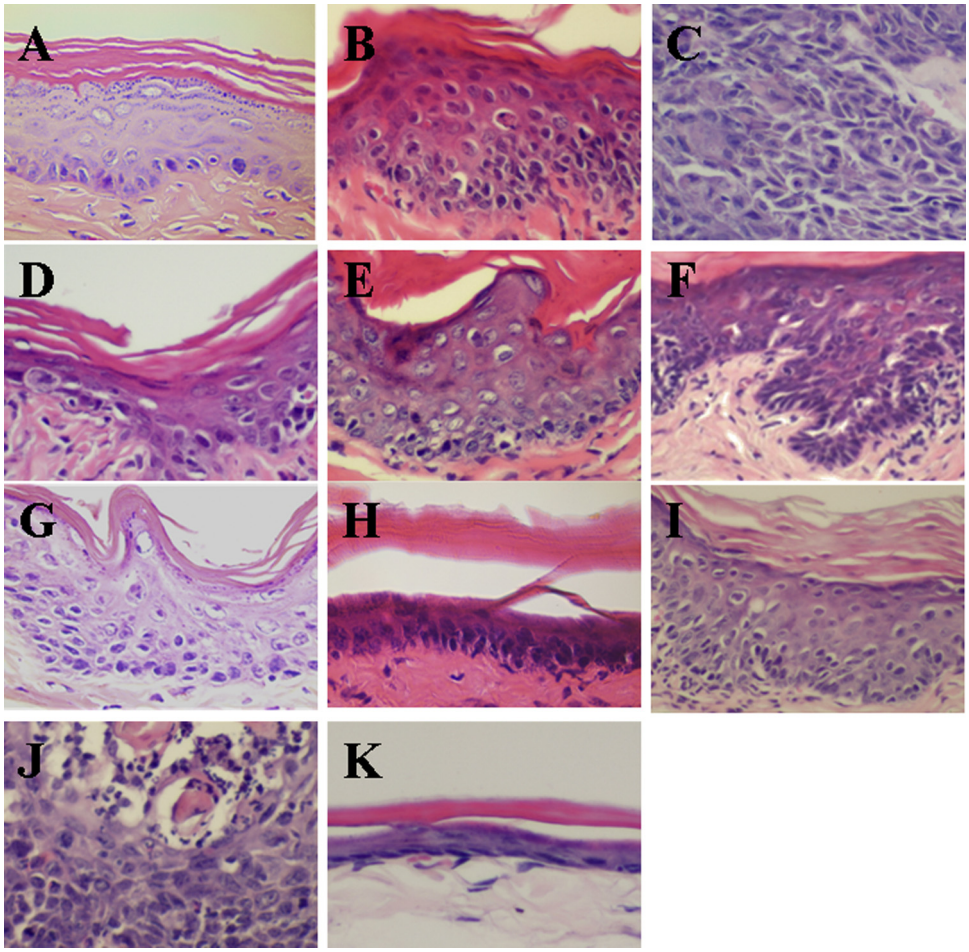

Fig. 1. Representative hematoxylin & eosin stainings of groups 1–10 and 13 (×200).
A: Group 1: HK and EH; B: Group 2: mild ED; C: Group 3: well-differentiated SCC; D: Group 4: mild ED; E: Group 5: moderate ED; F: Group 6: well-differentiated SCC; G: Group 7: mild ED; H: Group 8: mild ED; I: Group 9: severe ED; J: Group 10: well-differentiated SCC; K: Group 13: no obvious histopathological changes. HK: hyperkeratosis (similar finding in groups 11, 12); EH: epithelial hyperplasia; ED: epithelial dysplasia; SCC: squamous cell carcinoma

Table 4
Labeling index (LI, mean ± standard deviation) of PCNA, apoptotic index (AI, mean ± standard deviation) of TUNEL assay, and grades of survivin, XIAP, iNOS, COX-2, and poly (ADP-ribose) polymerase (PARP) expressions in each group.

Group	PCNA LI[a,b,c]	TUNEL AI[a,c]	Survivin[a,b,c]	XIAP[a,b,c]	iNOS[a,c]	COX−2[a,b,c]	Cytochrome C[a,b,c]	PARP[a,b,c]
1	32.60 ± 5.81	22.40 ± 3.85	1–2	1–2	1–2	2	3	3
2	43.40 ± 7.57	29.00 ± 3.94	2–3	2–3	2	2–3	2–3	2–3
3	61.45 ± 3.62	23.82 ± 2.64	3–4	3–4	3–4	3–4	2	2
4	32.40 ± 9.29	28.00 ± 2.55	1–2	1–2	1–2	1–3	2–3	2–3
5	36.25 ± 3.30	29.71 ± 3.25	1–2	1–2	1–2	1–3	2–3	2–3
6	39.95 ± 6.79	34.56 ± 4.84	2–3	2–3	2–3	2–3	2–3	2–3
7	37.20 ± 7.92	29.80 ± 6.91	1–3	1–3	1–2	1–3	3–4	3–4
8	29.00 ± 3.61	33.00 ± 4.07	1–2	1–2	1–2	1–2	3–4	3–4
9	43.27 ± 5.75	34.91 ± 4.61	2–3	2–3	2–3	2–3	3–4	3–4
10	63.25 ± 5.34	25.71 ± 2.82	3–4	3–4	3–4	3–4	2	2
13	16.80 ± 3.42	12.20 ± 1.92	0	0	0	0	2–3	2–3

[a] Significant differences between groups 1, 2, 3, and 13 by ANOVA followed by the Tukey-Kramer method ($P < 0.05$).
[b] Significant differences between groups 2, 5, and 8 by ANOVA followed by the Tukey-Kramer method ($P < 0.05$).
[c] Significant differences between groups 3, 6, 9, and 10 by ANOVA followed by the Tukey-Kramer method ($P < 0.05$).

(SED), carcinoma *in situ* (CIS), and SCC. The degree of dysplasia was graded in accordance with the following criteria (Wright & Shear, 1985): (i) MiED, dysplastic alterations limited to the lower third of the buccal epithelium; (ii) MoED, dysplastic changes noted for up to two thirds of the thickness of the oral epithelium; and (iii) SED, dysplastic cells observed within more than two thirds but less than the whole thickness of the oral epithelium. If dysplastic cells were observed within the entire thickness of the oral epithelium, CIS was designated. If one specimen contained more than one category of the above-mentioned histopathological findings, the most serious category was considered. The labeling index for PCNA and the apoptotic index for TUNEL assay were expressed as the number of cells with positive staining per 100 counted cells. A semi-quantitative scale was used to evaluate the expressions of

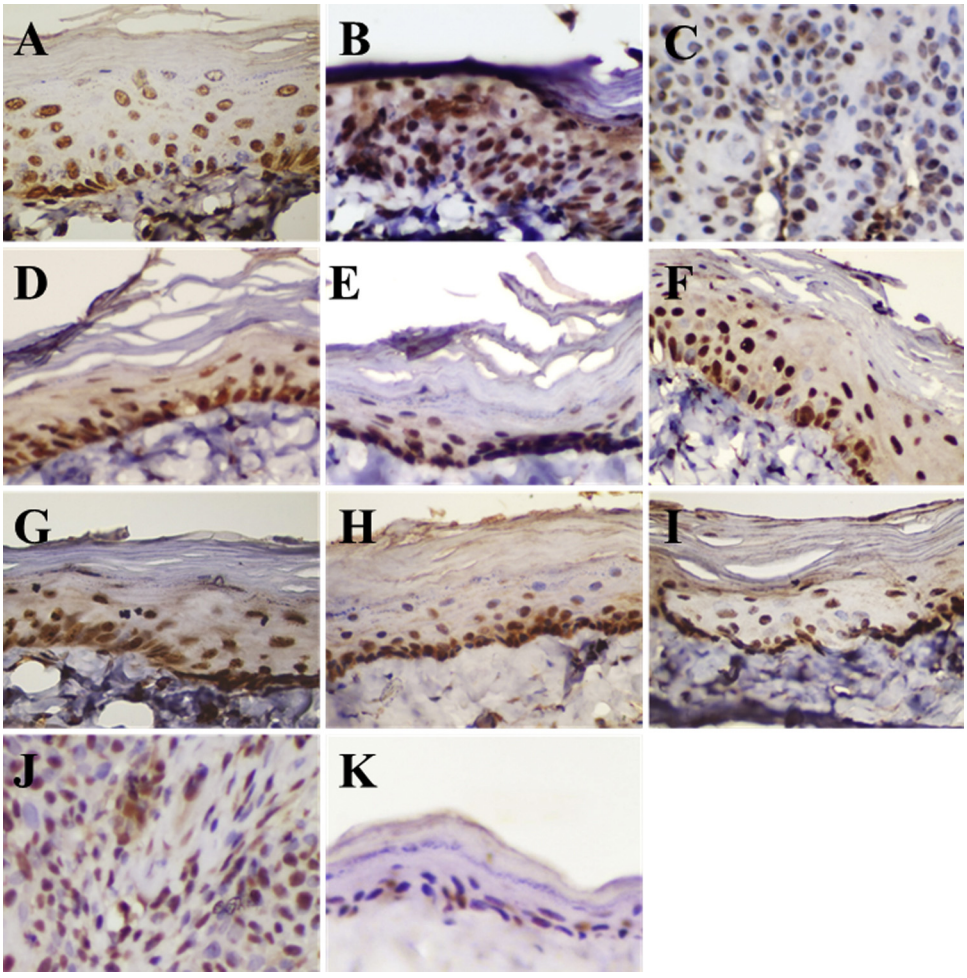

Fig. 2. PCNA expressions of groups 1–10 and 13 (labeled streptavidin-biotin method, ×200).
Nuclear stainings were observed in the basal layer of the untreated group (K), with the distribution of the positive cells extended towards the surface of the dysplastic and malignant epithelium (A–J).
A: group 1; B: group 2; C: groups 3; D: group 4; E: group 5; F: group 6; G: group 7; H: group 8; I: group 9; J: group 10; K: group 13 (similar finding in groups 11, 12).

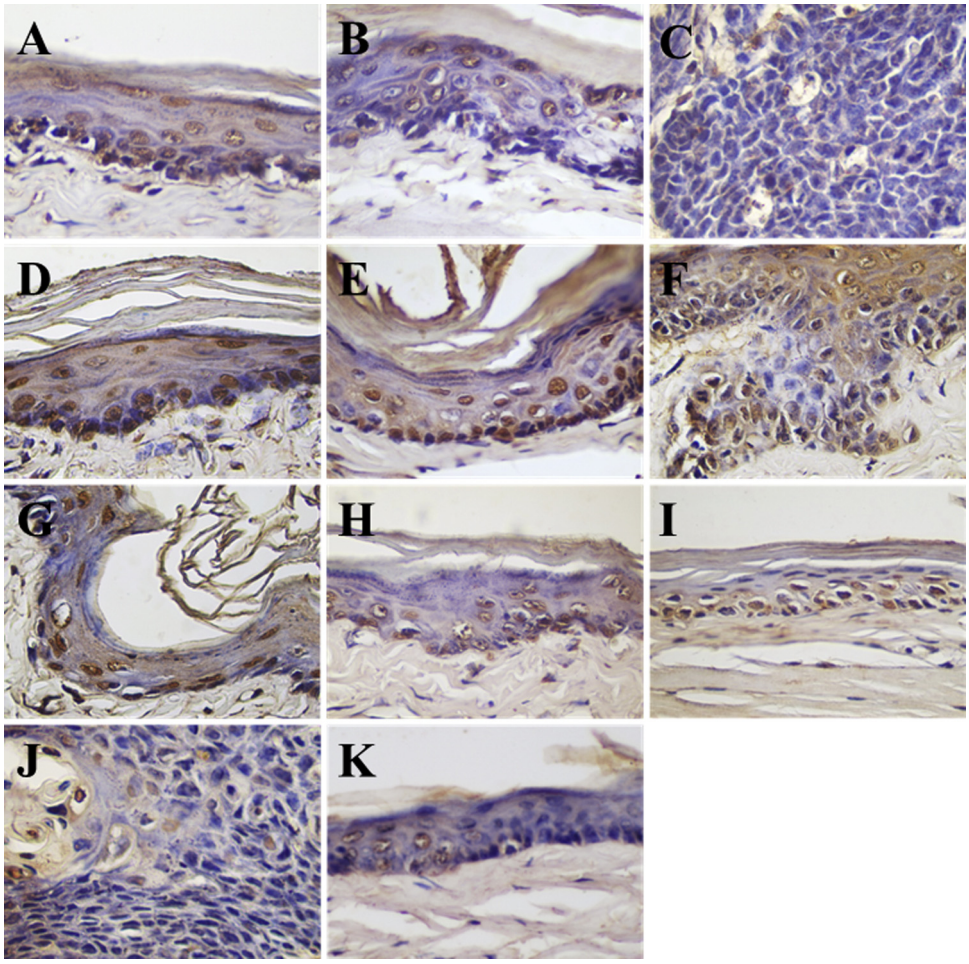

Fig. 3. Results of TUNEL assays of groups 1–10 and 13 (×200).
Nuclear positive cells were rarely observed in the basal layer of normal epithelium (K), but nuclear stainings were distributed throughout the dysplastic and malignant epithelium (A–J). Nuclear stainings were observed in the basal layer of the untreated group (K), with the distribution of the positive cells extended towards the surface of the dysplastic and malignant epithelium (A–J).
A: group 1; B: group 2; C: groups 3; D: group 4; E: group 5; F: group 6; G: group 7; H: group 8; I: group 9; J: group 10; K: group 13 (similar finding in groups 11, 12).

survivin, XIAP, iNOS COX-2, cytochrome C, and PARP. The extent of staining was graded as follows: 0, <10%; 1, 10–24%; 2, 25–49%; 3, 50–74%; and 4, >75%.

2.8. Statistical analyses

The quantitative differences in tumor number, tumor volume, tumor burden, labeling index, apoptotic index, and scores of survivin, XIAP, iNOS, COX-2, cytochrome C, and PARP expressions between groups were analyzed using analysis of variance (ANOVA) followed by the Tukey-Kramer method or Wilcoxon test where appropriate. The histopathological analyses, incidence of SCC, and incidence of dysplasia of each group were compared using the Chi-square test. A P value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Gross observation

A representative gross specimen of each group is shown in Supplementary Fig. S1. The means and standard deviations of weight, tumor number, tumor volume, and tumor burden are shown in Table 2. No apparent change was observed in both pouches of the animals in the control group. Thickened mucosa with whitish color and rough surfaces were noted in the pouches of the animals that underwent topical application of DMBA for 4 weeks (groups 1, 4, and 7) and 9 weeks (groups 2, 5, and 8). No tumorous growths were found. Two pouches had ulcerative lesions in groups 4 and 7. Tumorous growths were observed in the pouches of the animals treated with topical application of DMBA for 12 weeks (groups 3, 6, 9, and 10). In groups 3 and 10, tumors were found in all pouches, with a mean tumor number of 7.22 and 5.06, respectively. Although the mean tumor number of group 10 was smaller, the mean tumor volume and tumor burden of group 10 (68.33 mm^3, 273.94 mm^3) were greater than those of group 3 (24.97 mm^3, 222.64 mm^3), and there were no significant differences in tumor number, tumor volume, or tumor burden between groups 3 and 10. Tumors were observed in the animals of group 6 (15/28, 53.47%) and group 9 (8/16, 50%). The mean tumor number, tumor volume, and tumor burden of groups 6 and 9 were significantly decreased compared with groups 3 and 10 ($P < 0.0001$; $P = 0.0020$; $P < 0.0001$). However, comparing the data of group 6 with those of group 9 by the Wilcoxon test, there were

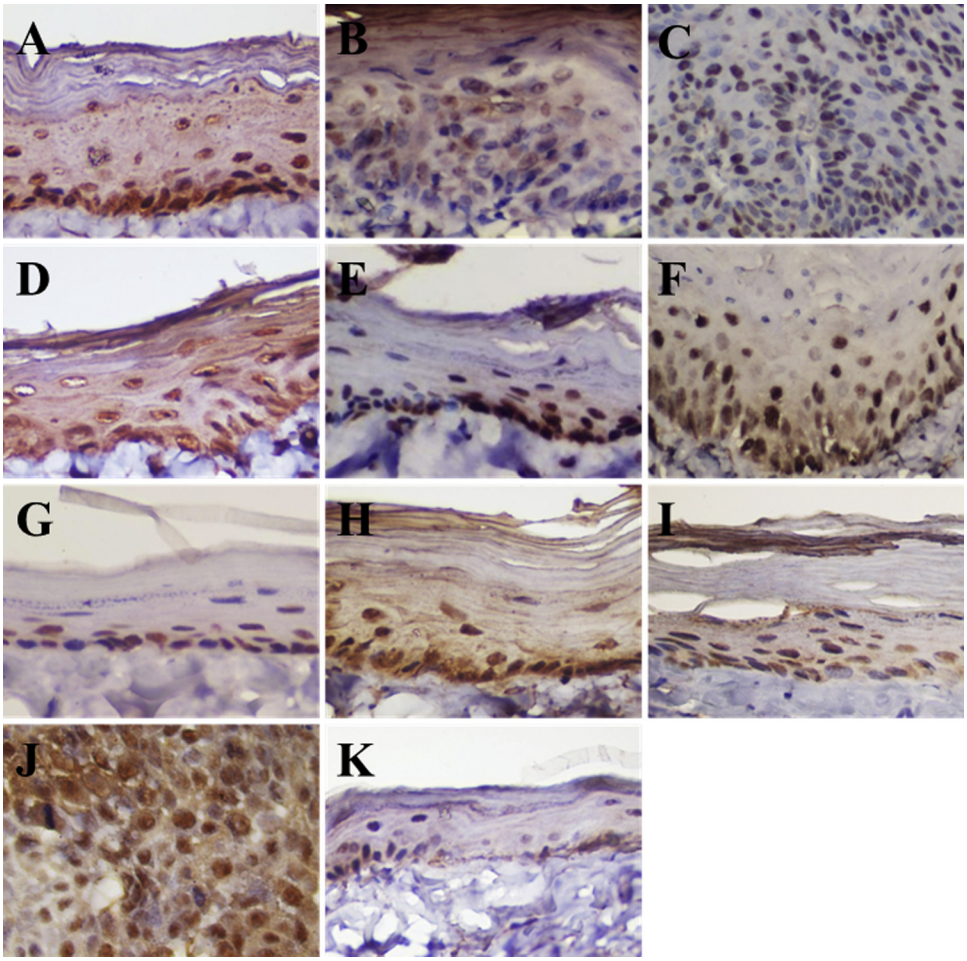

Fig. 4. Expressions of survivin protein of groups 1–10 and 13 (labeled streptavidin-biotin method, ×200).
Negative stainings were observed in the untreated group (K), with both cytoplasmic and nuclear stained positive cells extended towards the surface of the dysplastic and malignant epithelium (A–J). Nuclear stainings were observed in the basal layer of the untreated group (K), with the distribution of the positive cells extended towards the surface of the dysplastic and malignant epithelium (A–J).
A: group 1; B: group 2; C: groups 3; D: group 4; E: group 5; F: group 6; G: group 7; H: group 8; I: group 9; J: group 10; K: group 13 (similar finding in groups 11, 12).

no significant differences in tumor number, tumor volume, or tumor burden. TSL extract administration prior to tumor induction greatly decreased the incidence, number, volume, and burden of tumors; on the other hand, little inhibitory effect was observed when TSL extract was applied after tumor induction.

3.2. Histopathological observation

The histopathological status, the incidence of SCC, and the incidence of dysplasia of groups 1–10 and 13 are summarized in Table 3. Fig. 1 shows the histopathological findings for each group. The pouches of the animals in the control groups demonstrated no obvious histopathological changes (Fig. 1K). In the carcinogenic group, in addition to hyperkeratosis and epithelial hyperplasia, 80% of the pouches treated with DMBA for 4 weeks (group 1, Fig. 1A) exhibited mild dysplastic changes, and all pouches treated with DMBA for 9 weeks (group 2, Fig. 1B) showed moderate to severe dysplastic changes. The histopathological status differed significantly between groups 1 and 2 ($P = 0.0002$), but the incidence of dysplasia did not ($P = 0.1360$). The pouches treated with DMBA for 12 weeks (group 3, Fig. 1C) all developed well-differentiated and exophytic SCC. As the time of DMBA painting was prolonged, the severity of dysplasia increased, and finally, the premalignant lesions transformed into SCC. In the preventive group, the incidence of dysplasia in groups 4 (Fig. 1D) and 7 (Fig. 1G) (the animals that underwent DMBA painting for 4 weeks) were 60% and 80%, respectively. No significant differences were observed in the histological status and the severity of the dysplastic lesions between groups 1, 4 and 7. The effect of TSL extract was obscure in the early stage of HBP carcinogenesis. The incidences of dysplasia were significantly decreased in groups 5 (71.43%) (Fig. 1E) and 8 (37.5%) (Fig. 1H), compared with group 2 (100%), and so was the severity of dysplastic lesions. The decreases in the incidence and severity of dysplasia between groups 2 and 8 were statistically significant ($P = 0.0033$ and $P = 0.0052$, respecitvely). Between groups 2 and 5, only the decrease in the severity of dysplasia was statistically significant ($P = 0.0469$). Between groups 5 and 8, no significant differences were observed. The preventive effect of TSL extract became more apparent as HBP carcinogenesis progressed. The incidences of SCC in groups 6 (53.47%) (Fig. 1F) and 9 (50%) (Fig. 1I) were significantly reduced ($P = 0.0006$; $P = 0.0006$) compared with those of group 3 (100%). Comparing the

incidence and histopathologic status between groups 3 and 10 (Fig. 1J) and between groups 6 and 9 indicated no significant differences.

3.3. Immunohistochemical analysis

Table 4 presents the analyzed results for the labeling index of PCNA, apoptotic index of TUNEL assay, and the grades of survivin, XIAP, iNOS, COX-2, cytochrome C, and PARP expressions of each group. The microscopic characteristics are shown in Figs. 2–9.

3.3.1. PCNA

In the untreated group, the PCNA positive cells were located in the basal layer and demonstrated nuclear staining (Fig. 2K). With the progression of HBP carcinogenesis, the distribution of PCNA-positive cells extended toward the surface of the epithelium. The labeling index of PCNA increased throughout HBP carcinogenesis. Significant differences between normal epithelium (group 13, Fig. 2K), dysplastic epithelium (groups 1, 2, Fig. 2A, 5B), and malignant epithelium (group 3, Fig. 2C) ($P < 0.0001$) were observed. TSL extract administration prior to tumor induction resulted in significant decreases in the labeling indexes of groups 5 (Fig. 2E), 8 (Fig. 2H), 6 (Fig. 2F), and 9 (Fig. 2I). No significant changes were detected in the early stage of carcinogenesis (groups 4, 7, Fig. 2D, 2G), and the labeling index of the therapeutic group (group 10, Fig. 2J) was similar to that of group 3.

3.3.2. TUNEL assay

The apoptotic index of the TUNEL assay significantly increased from normal (Fig. 3K) to dysplastic epithelium (Fig. 3A, B). Although a drop in the apoptotic index between dysplastic and malignant epithelia (Fig. 3C) was observed, the apoptotic index of malignant epithelium was still higher than that of normal epithelium, and the difference was significant. Contrary to the PCNA-positive cells, TUNEL positive cells were rarely found in the basal layer of normal epithelium, and demonstrated nuclear staining. In dysplastic and malignant epithelia, the TUNEL-positive cells were distributed throughout the epithelium. The apoptotic index was significantly increased in groups 6 (Fig. 3F) and 9 (Fig. 3I), relative to group 3 (Fig. 3C). The apoptotic indices in other groups that underwent TSL extract administration showed no significant increases. The therapeutic group (Fig. 3J) also failed to show a significant difference compared with group 3.

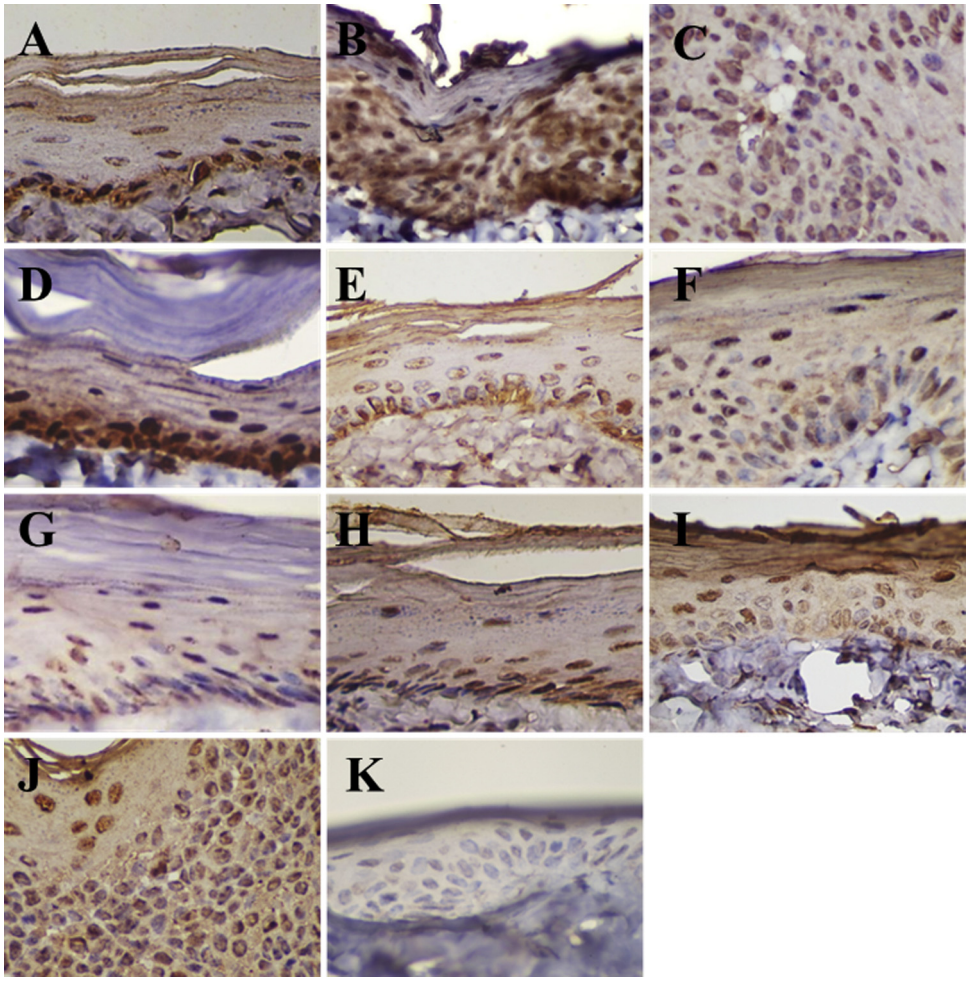

Fig. 5. Expressions of XIAP protein of groups 1–10 and 13 (labeled streptavidin-biotin method, ×200).
Negative stainings were observed in the untreated group (K), with both cytoplasmic and nuclear stained positive cells extended towards the surface of the dysplastic and malignant epithelium (A–J). Nuclear stainings were observed in the basal layer of the untreated group (K), with the distribution of the positive cells extended towards the surface of the dysplastic and malignant epithelium (A–J).
A: group 1; B: group 2; C: groups 3; D: group 4; E: group 5; F: group 6; G: group 7; H: group 8; I: group 9; J: group 10; K: group 13 (similar finding in groups 11, 12).

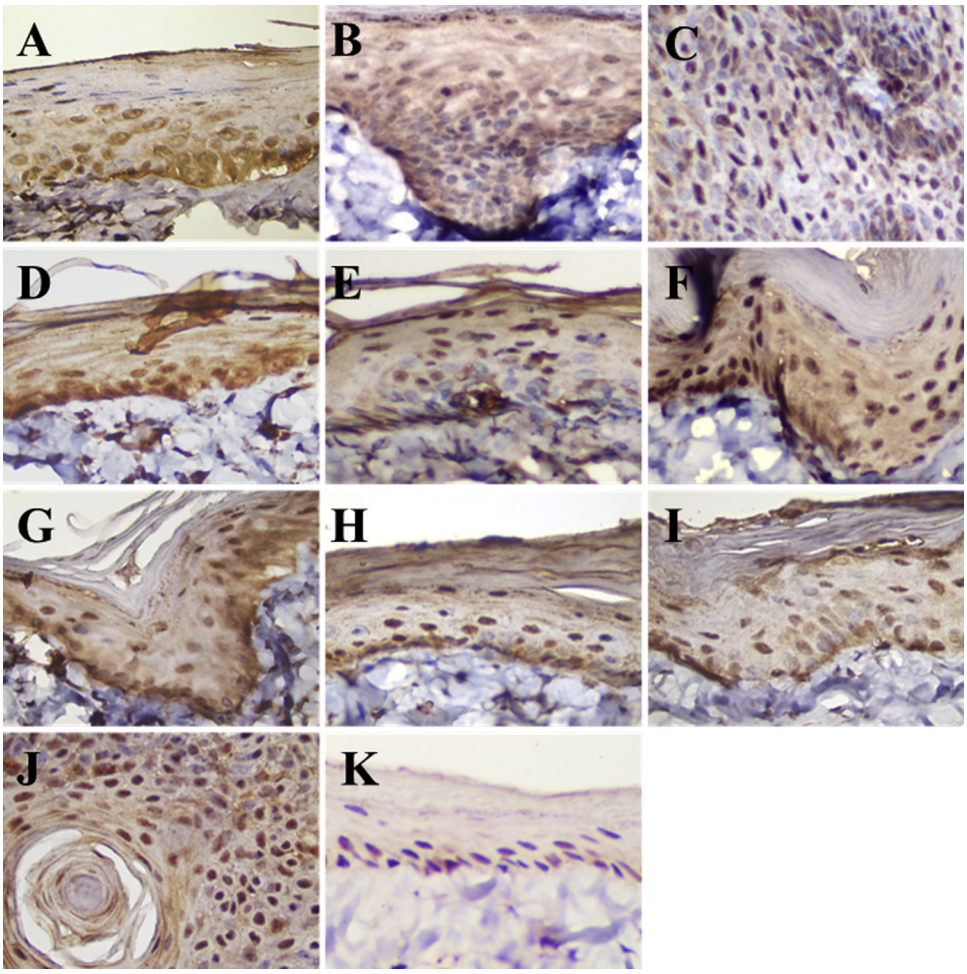

Fig. 6. Expressions of iNOS protein of groups 1–10 and 13 (labeled streptavidin-biotin method, ×200).
Negative stainings were observed in the untreated group (K), with both cytoplasmic and nuclear stained positive cells extended towards the surface of the dysplastic and malignant epithelium (A–J). Nuclear stainings were observed in the basal layer of the untreated group (K), with the distribution of the positive cells extended towards the surface of the dysplastic and malignant epithelium (A–J).
A: group 1; B: group 2; C: groups 3; D: group 4; E: group 5; F: group 6; G: group 7; H: group 8; I: group 9; J: group 10; K: group 13 (similar finding in groups 11, 12).

3.3.3. Survivin, XIAP, iNOS, and COX-2 expression levels

The untreated control groups showed no expression of survivin (Fig. 4K), XIAP (Fig. 5K), iNOS (Fig. 6K), or COX-2 (Fig. 7K). As HBP carcinogenesis progressed, the grades of survivin, XIAP, iNOS, and COX-2 expressions significantly increased (Table 4). Both cytoplasmic and nuclear staining was observed (Figs. 4–7). In the early stage of carcinogenesis (groups 1, 4 and 7), none of these proteins was found to exhibit a significant decrease in expression in groups 4 and 7 (Table 4). In the late stage of carcinogenesis (groups 2, 5, and 8), the expressions of survivin, XIAP, and COX-2 were significantly decreased in the preventive groups (Table 4). The decreases in the expressions of survivin, XIAP, iNOS, and COX-2 in the preventive groups became more prominent at the cancer stage (groups 3, 6, 9, and 10) (Table 4).

3.3.4. Cytochrome C and PARP expressions

Obvious cytochrome C (Fig. 8K) and PARP (Fig. 9K) expressions were noted for the untreated control groups. The grades of cytochrome C and PARP expressions were significantly decreased as HBP carcinogenesis progressed (Table 4). Cytoplasmic staining was noted for cytochrome C expression (Fig. 8), whereas nuclear staining was observed for PARP expression (Fig. 9). In the early stage of carcinogenesis (groups 1, 4 and 7), cytochrome and PARH expressions were determined to be significantly increased in groups 4 and 7 (Table 4). In the late stage of carcinogenesis (groups 2, 5, and 8), the expressions of cytochrome C and PARP were also significantly elevated in the preventive groups (Table 4).

4. Discussion

In the majority of studies examining cancer chemoprevention *in vitro*, cancer cell lines have been used (Chang et al., 2006; Chen et al., 2009; Chia et al., 2010; Han et al., 2005; Wang et al., 2010; Yang et al., 2006; Yang et al., 2010). In fact, chemopreventive agents have been used to treat the cancer cells, not to prevent carcinogenesis. Therefore, a DMBA-induced HBP carcinogenesis animal model was used, and the chemopreventive effect on premalignant lesions was evaluated in the present study. Reviewing the literature related to chemoprevention of oral cancer using animal models (Chandra Mohan et al., 2006; Harish Kumar et al., 2010; Manoharan et al., 2009; Nishimura et al., 2004; Sun et al., 2008), chemopreventive agents have been administered to animals at the same time that carcinogens were applied. This protocol could not be used to evaluate the efficacy of the use of

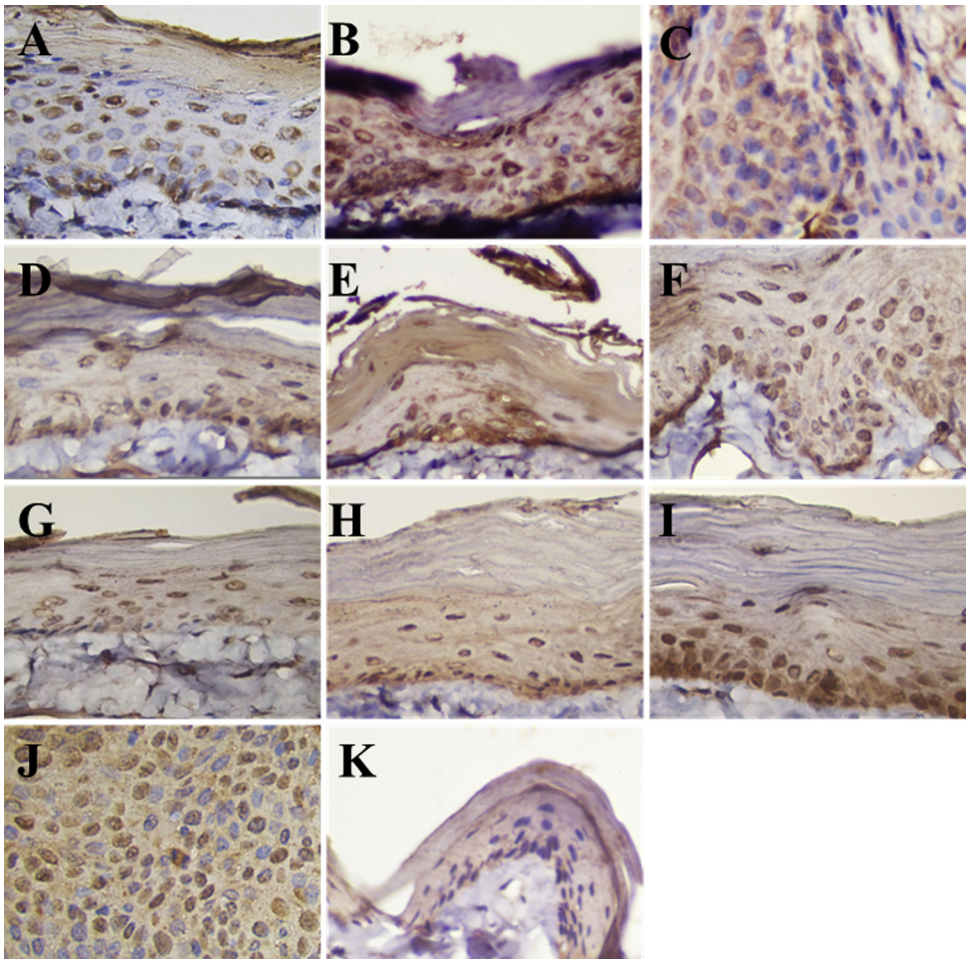

Fig. 7. Expressions of COX-2 protein of groups 1–10 and 13 (labeled streptavidin-biotin method, ×200).
Negative staining was observed in the untreated group (K), with both cytoplasmic and nuclear stained positive cells extended towards the surface of the dysplastic and malignant epithelium (A-J). Nuclear stainings were observed in the basal layer of the untreated group (K), with the distribution of the positive cells extended towards the surface of the dysplastic and malignant epithelium (A-J).
A: group 1; B: group 2; C: groups 3; D: group 4; E: group 5; F: group 6; G: group 7; H: group 8; I: group 9; J: group 10; K: group 13 (similar finding in groups 11, 12).

chemopreventive agents before carcinogen administration. Therefore, we used a different study design in terms of the timing of administration of the chemopreventive agent. According to previous research on TSL extract (Chang et al., 2006; Chia et al., 2010; Hseu et al., 2011; Yang et al., 2010), its mechanisms of cancer chemoprevention include suppressing growth and inducing apoptosis of cancer cells. In order to prove the theory and to provide a possible direction for future molecular study, proliferative and apoptosis-related markers, including PCNA, survivin, XIAP, iNOS, COX-2, cytochrome C, PARP, and TUNEL assay, were examined.

The changes in histopathological status during DMBA-induced HBP carcinogenesis and their timing in the present study were similar to the results of Kim et al. (2004), Lajolo et al. (2008), and Nishimura et al. (2004). Epithelial hyperplasia was observed after DMBA painting for 2 weeks. Mild epithelial dysplasia was present after DMBA painting for 4 weeks. After DMBA painting for 8 weeks, severe epithelial dysplasia developed. Finally, SCC formed after DMBA painting for 10–12 weeks. The activities of PCNA, survivin, XIAP, iNOS, and COX-2 were increased with the progression of HBP carcinogenesis. The strongest expressions were observed in malignant epithelium. The trend observed in the present study was consistent with the results of our previous studies of survivin, XIAP, and iNOS (Chen & Lin, 2000; Hsue et al., 2008). Comparing the labeling index (LI) of PCNA in our study with the results of studies by Grawish, Zaher, Gaafar, and Nasif (2010), Kobayashi et al. (1995), and Tsuji et al. (1992), there were minor differences in the PCNA LI value, but the trend and the conclusion were the same. In the current study, both expressions of cytochrome C (a specific apoptotic marker that initiates the caspase activation pathway (Jiang & Wang, 2004)), and PARP (also a specific marker of apoptosis (Kockx et al., 1998)) demonstrated a progressive decline from normal to dysplastic epithelium, and further to malignant epithelium. On the other hand, the results of the TUNEL assay, which is less specific for apoptosis, showed a rise from normal to dysplastic epithelium, followed by a drop from dysplastic epithelium to malignant epithelium. Kohno et al. (2002) reported a similar change of the AI of the TUNEL assay. Survivin and XIAP are also associated with the regulation of apoptosis (Hsue et al., 2008). Increasing expressions of survivin and XIAP will inhibit apoptotic activity. The grades of survivin and XIAP expressions increased from normal to dysplastic epithelium, so the apoptotic activity should reduce, and as a consequence, the expression of cytochrome C and PARP was respectively decreased, as demonstrated

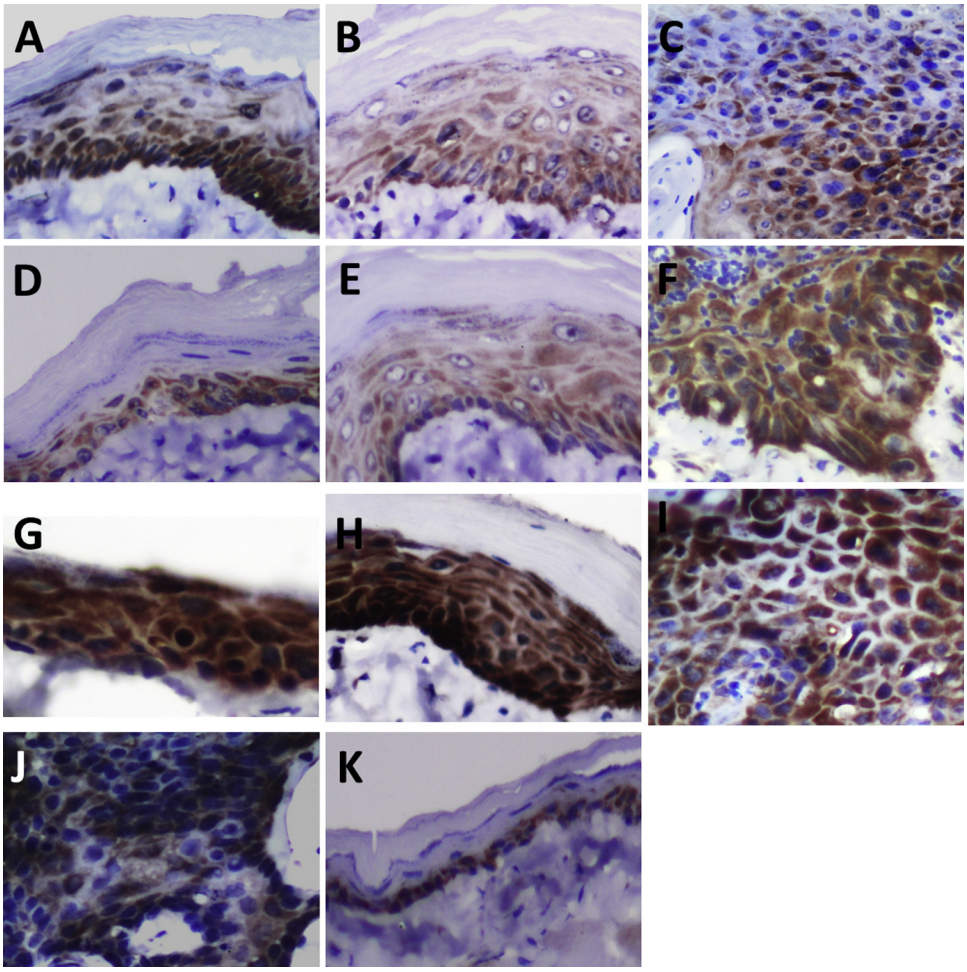

Fig. 8. Expressions of cytochrome C protein of groups 1–10 and 13 (labeled streptavidin-biotin method, ×200).
Cytoplasmic stainings were observed in the lower layer of the untreated group (K), with the distribution of the positive cells extended towards the surface of the dysplastic and malignant epithelium (A-J).
A: group 1; B: group 2; C: groups 3; D: group 4; E: group 5; F: group 6; G: group 7; H: group 8; I: group 9; J: group 10; K: group 13 (similar finding in groups 11, 12).

in the current study. On the contrary, the AI of the TUNEL assay was increased, though not significantly. The complicated biologic network that regulates apoptotic activity might be one reason for this discrepancy. The distribution of survivin in cells might be another reason. While cytoplasmic survivin is known to trigger apoptosis evasion, nuclear survivin is known to promote intrinsic apoptosis (Thiyagarajan et al., 2012). In our study, both cytoplasmic and nuclear staining was present. Thus, the roles of survivin and XIAP might change due to time and distribution. Comparing the grades of XIAP, iNOS, COX-2, cytochrome C, PARP, and TUNEL assay between groups 2 and 3 indicated no significant differences suggesting that it is difficult to separate mild, moderate, and severe epithelial dysplasia, not only according to the histopathological findings but also the molecular changes.

The obvious decreases in tumor number, tumor volume, tumor burden, and incidence of SCC in groups 6 and 9 with TSL extract administration prior to topical application of DMBA for 12 weeks indicated that TSL extract exerted a chemopreventive effect on HBP carcinogenesis. However, no significant changes in the tumor number, tumor volume, tumor burden, and incidence of SCC were observed in group 10, in which TSL extract was administered after cancer was induced. TSL extract failed to exert a therapeutic influence in the treatment of oral cancer. The tumor volume and burden of group 10 were larger than those of group 3. The animals in group 3 were sacrificed immediately after the formation of tumors, while the animals in group 10 underwent TSL extract administration for 4 weeks after the formation of tumors. The tumors in the animals of group 10 continued to grow because the duration of tumor growth in group 10 was 4 weeks longer than the duration of tumor growth in group 3. There were no significant differences between the results of group 6 and those of group 9. Continued administration of TSL extract after tumor formation did not improve the chemopreventive effect. The crucial timing of TSL administration was prior to animals coming into contact with the carcinogen. The decreased expressions of PCNA and increased expressions of cytochrome C, PARP, and TUNEL assay in groups 6 and 9 suggested that the chemopreventive effect of TSL extract was accomplished by inhibiting tumor cell proliferation and inducing tumor cell apoptosis. The decreases in the grades of survivin, XIAP, iNOS, and COX-2 expressions also supported the above suggestion. Furthermore, this suggestion was consistent with the conclusions of previous *in vitro* studies (Chang et al., 2006; Chia et al., 2010; Wang et al., 2010; Yang et al., 2010).

The chemopreventive effect of TSL extract was obscure in the early stage of carcinogenesis. Groups 1, 4, and 7 did not exhibit significant differences in histological status, incidence of dysplastic

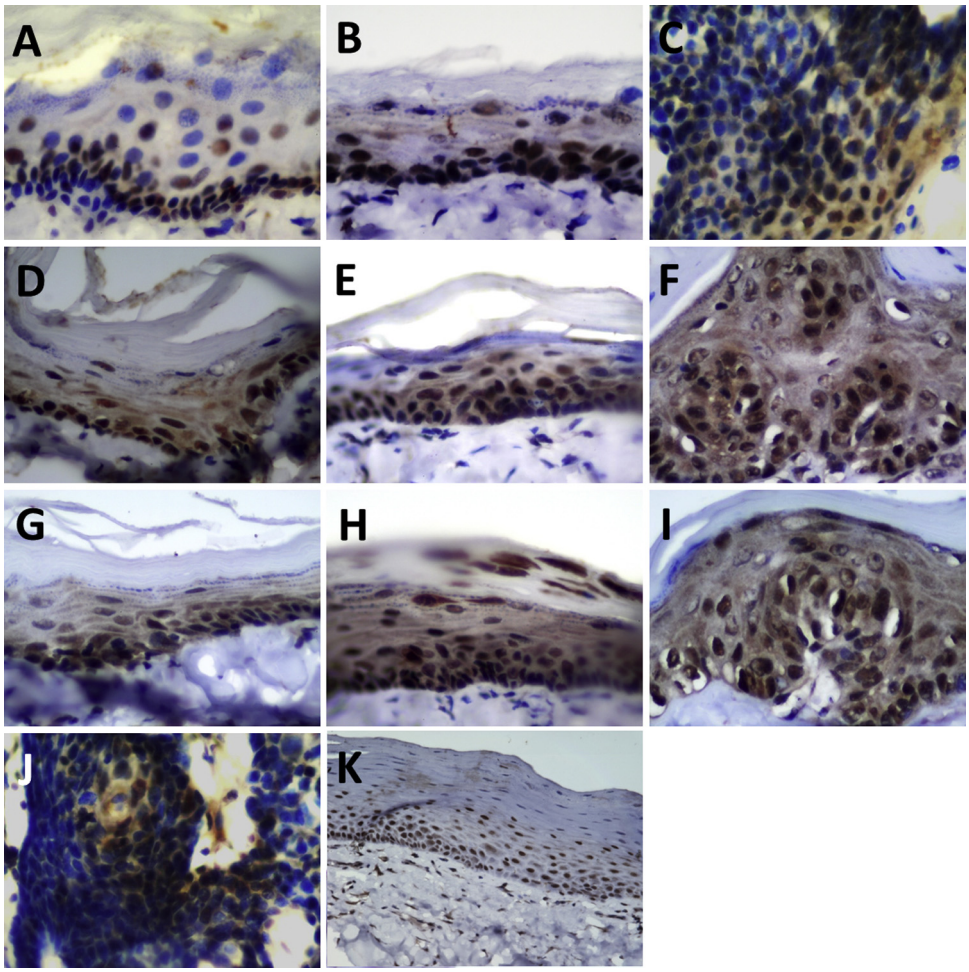

Fig. 9. Expressions of PARP protein of groups 1–10 and 13 (labeled streptavidin-biotin method, ×200).
Nuclear stainings were observed in the lower layer of the untreated group (K), with the distribution of the positive cells extended towards the surface of the dysplastic and malignant epithelium (A-J).
A: group 1; B: group 2; C: groups 3; D: group 4; E: group 5; F: group 6; G: group 7; H: group 8; I: group 9; J: group 10; K: group 13 (similar finding in groups 11, 12).

lesions, or grades of immunohistochemical staining. As carcinogenesis progressed to the late stage, significant decreases in the incidence and severity of dysplastic lesions were observed in group 8. Although the incidence of dysplasia in group 5 did not reduce significantly, the severity of dysplasia was significantly improved. The incidence of dysplasia in group 7 with TSL extract administration before and during topical application of DMBA for 4 weeks was 80%. It is interesting to note that in group 8, with TSL extract administration before and during topical application of DMBA for 9 weeks, the incidence of dysplasia dropped to 37.5%. This result indicated that TSL extract might have the ability not only to prevent but also to reverse the carcinogenic process. The expressions of PCNA, survivin, XIAP, and COX-2 were significantly decreased between groups 2 and 8, as well as between groups 3 and 9, while the expressions of cytochrome C and PARP were significantly increased between groups 2 and 8, as well as between groups 3 and 9. Hence, the decreased expressions of PCNA and COX-2 implied a reduction in proliferation, and the inhibitory effect of TSL extract due to the increased apoptotic activity, as indicated by the increased expressions of cytochrome C and PARP, occurred between the early and late stage of carcinogenesis.

Because TSL extract was administered by gavage in the current study, the chemopreventive activity of TSL would be acquired by systemic route only. So, it is essential to perform the chemical analysis of TSL extract to recognize the bioactive substances present in the extract responsible to elicit the chemopreventive effect on oral carcinogenesis. A previous study has been conducted analyzing the major component of TSL extract on oral cancer cells (Chia et al., 2010). In this previous study (Chia et al., 2010), the authors not only demonstrated that TSL extract could induce cell death in oral cancer cells and not in normal oral epithelial cells but also identified that gallic acid (3,4,5-trihydroxybenzoic acid) being purified from the TSL extract could induce anti-neoplastic activity by up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes producing cell death. Although not performed in the present study, chemical analyses of TSL extract indicate that the bioactive compounds correlate with the biological activity of oral cancer cell lines. Taking into consideration the above-mentioned study (Chia et al., 2010) and the present findings regarding up-regulation of pro-apoptotic proteins (cytochrome and PARP) as well as down-regulation of anti-apoptotic proteins (survivin and XIAP), it may be assumed that gallic acid could also be the putative bioactive compound associated with the chemopreventive activity of TSL extract demonstrated in the current study.

5. Conclusions

The present study demonstrated that TSL extract has chemopreventive potential in DMBA-induced HBP carcinogenesis due to

the anti-proliferative and apoptosis-inducing abilities of TSL. In addition, TSL resulted in a better outcome in terms of chemoprevention than in the treatment of oral cancer. These findings suggest that TSL extract might be a promising candidate for the prevention of oral cancer.

Conflict of interest

None declared.

Acknowledgments

The authors are grateful to Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung, Taiwan, (grant # kmtth-99-008) for financial support. We are also grateful to health and welfare surcharge of tobacco products, the Ministry of Health and Welfare, Taiwan, Republic of China (MOHW103-TD-B-111-05; MOHW105-TDU-B-212-134007).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2016.06.015.

References

Baldasquin-Caceres, B., Gomez-Garcia, F. J., López-Jornet, P., Castillo-Sanchez, J., & Vicente-Ortega, V. (2014). Chemopreventive potential of phenolic compounds in oral carcinogenesis. *Archives of Oral Biology*, *59*(10), 1101–1107.

Brinkman, B., & Wong, T. W. (2006). Disease mechanism of biomarkers of oral squamous cell carcinoma. *Current Opinion in Oncology*, *18*(3), 228–233.

Chandra Mohan, K. V., Devaraj, H., Prathiba, D., Hara, Y., & Nagini, S. (2006). Antiproliferative and apoptosis inducing effect of lactoferrin and black tea polyphenol combination on hamster buccal pouch carcinogenesis. *Biochimica et Biophysica Acta*, *1760*(10), 1536–1544.

Chang, H. L., Hsu, H. K., Su, J. H., Wang, P. H., Chung, Y. F., Chia, Y. C., et al. (2006). The fractionated *Toona sinensis* leaf extract induces apoptosis of human ovarian cancer cells and inhibits tumor growth in a murine xenograft model. *Gynecologic Oncology*, *102*(2), 309–314.

Chen, Y. K., & Lin, L. M. (2000). Immunohistochemical expression of inducible nitric oxide synthase in DMBA-induced hamster buccal pouch carcinogenesis. *Oral Oncology*, *36*(2), 221–224.

Chen, H. M., Wu, Y. C., Chia, Y. C., Chang, F. R., Hsu, H. K., Hsieh, Y. C., et al. (2009). Gallic acid, a major component of *Toona sinensis* leaf extracts, contains a ROS-mediated anti-cancer activity in human prostate cancer cells. *Cancer Letters*, *286*(2), 161–171.

Chia, Y. C., Rajbanshi, R., Calhoun, C., & Chiu, H. (2010). Anti-neoplastic effects of gallic acid, a major component of *Toona sinensis* leaf extract, on oral squamous carcinoma cells. *Molecules*, *15*(11), 8377–8389.

Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., et al. (2013). *GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC cancer base no. 11*. Lyon, France: International Agency for Research on Cancer.

Grawish, M. E., Zaher, A. R., Gaafar, A. I., & Nasif, W. A. (2010). Long-term effect of *Spirulina platensis* extract on DMBA-induced hamster buccal pouch carcinogenesis (immunohistochemical study). *Medical Oncology*, *27*(1), 20–28.

Han, C., Ding, H., Casto, B., Stoner, G. D., & D'Ambrosio, S. M. (2005). Inhibition of the growth of premalignant and malignant human oral cell lines by extracts and components of black raspberries. *Nutrition and Cancer*, *51*(2) 207–207.

Harish Kumar, G., Vidya Priyadarsini, R., Vinothini, G., Vidjaya Letchoumy, P., & Nagini, S. (2010). The neem limonoids azadirachtin and nimbolide inhibit cell proliferation and induce apoptosis in an animal model of oral oncogenesis. *Investigational New Drugs*, *28*(4), 392–401.

Hong, W. K., Endicott, J., Itri, L. M., Doos, W., Batsakis, J. G., Bell, R., et al. (1986). 13-*cis*-retinoic acid in the treatment of oral leukoplakia. *The New England Journal of Medicine*, *315*(24), 1501–1505.

Hseu, Y. C., Chen, S. C., Lin, H. W., Hung, D. Z., Lin, M. K., Kuo, Y. H., et al. (2011). Toona sinensis (leaf extracts) inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis in vascular endothelial cells. *Journal of Ethnopharmacology*, *134*(1), 111–121.

Hsue, S. S., Chen, Y. K., & Lin, L. M. (2008). Expression of survivin and XIAP for DMBA-induced hamster buccal pouch squamous cell carcinogenesis is associated with p53 accumulation. *Oral Oncology*, *44*(1), 43–49.

Jiang, X., & Wang, D. (2004). Cytochrome C-mediated apoptosis. *Annual Review of Biochemistry*, *73*, 87–106.

Kim, S. A., Ahn, S. G., Kim, D. K., Kim, S. G., Lee, S. H., Kim, J., et al. (2004). Sequential expression of inducible nitric oxide synthase and cyclooxygenase-2 during DMBA-induced hamster buccal pouch carcinogenesis. *In Vivo*, *18*(5), 609–614.

Kobayashi, I., Matsuo, K., Ozeki, S., Ohishi, M., Ishibashi, Y., & Sakai, H. (1995). The proliferative activity in oral epithelial dysplasia analyzed by proliferating cell nuclear antigen immunostaining and argyrophilic nuclear organizer region staining. *Human Pathology*, *26*(8), 907–913.

Kockx, M. M., Muhring, J., Knaapen, M. W., & de Meyer, G. R. (1998). RNA synthesis and splicing interferes with DNA in situ end labeling techniques used to detect apoptosis. *American Journal of Pathology*, *152*(4), 885–888.

Kohno, Y., Patel, V., Kim, Y., Tsuji, T., Chin, B. R., Sun, M., et al. (2002). Apoptosis, proliferation and p12 (doc-1) profiles in normal, dysplastic and malignant squamous epithelium of the Syrian hamster cheek pouch model. *Oral Oncology*, *38*(3), 274–280.

Lajolo, C., Giuliani, M., Sgambato, A., Majorano, E., Lucchese, A., & Capodiferro Favia, G. (2008). *N*-(4-hyproxyphenyl) all-trans-retinamide (4-HPR) high dose effect on DMBA-induced hamster oral cancer: a histomorphometric evaluation. *International Journal of Oral and Maxillofacial Surgery*, *37*(12), 1133–1140.

Lin, L. M., & Chen, Y. K. (1997). Diurnal variation of γ-glutamyl transpeptidase activity during DMBA-induced hamster buccal pouch carcinogenesis. *Oral Diseases*, *3*(3), 153–156.

Manoharan, S., Balakrishnan, S., Menon, V. P., Alias, L. M., & Reena, A. R. (2009). Chemopreventive efficacy of curcumin and piperine during 7,12-dimethylbenz [a]anthracene-induced hamster buccal pouch carcinogenesis. *Singapore Medical Journal*, *50*(2), 139–146.

McGurk, M., Chan, C., Jones, J., O'Reagan, E. O., & Sherriff, M. (2005). Delay in diagnosis and its effect on outcome in head and neck cancer. *British Journal of Oral and Maxillofacial Surgery*, *43*(4), 281–284.

Nishimura, N., Urade, M., Hashitani, S., Noguchi, K., Manno, Y., Takaoka, K., et al. (2004). Increased expression of cyclooxygenase (COX)-2 in DMBA-induced hamster cheek pouch carcinogenesis and chemopreventive effect of a selective COX-2 inhibitor celecoxib. *Journal of Oral Pathology and Medicine*, *33*(10), 614–621.

Sporn, M. B., Dunlog, N. M., Newton, D. L., & Smith, J. M. (1976). Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Federation Proceedings*, *35*(6), 1332–1338.

Sun, Z., Sood, S., Li, N., Yang, P., Newman, R. A., Yang, C. S., et al. (2008). Chemoprevention of 7,12-dimethylbenz[a]-anthracene (DMBA)-induced oral carcinogenesis in hamster cheek pouch by topical application of a dual inhibitor of epidermal growth factor receptor (EGFR) and ErbB2 tyrosine kinases. *Oral Oncology*, *44*(7), 652–657.

Thiyagarajan, P., Senthil Murugan, R., Kavitha, K., Anitha, P., Prathiba, D., & Nigini, S. (2012). Dietary chlorophyllin inhibits the canonical NF-κB signaling pathway and induces intrinsic apoptosis in a hamster model of oral oncogenesis. *Food and Chemical Toxicology*, *50*(3–4), 867–876.

Tsuji, T., Sasaki, K., Kimura, Y., Yamada, K., Mori, M., & Shinozaki, F. (1992). Measurement of proliferating cell nuclear antigen (PCNA) and its clinical application in oral cancers. *International Journal of Oral and Maxillofacial Surgery*, *21*(6), 369–372.

Wang, Y. C., Lin, K. H., Yang, C. J., Tsai, J. R., Hung, J. Y., Wang, P. H., et al. (2010). *Toona sinensis* extracts induced cell cycle arrest and apoptosis in the human lung large cell carcinoma. *Kaohsiung Journal of Medical Sciences*, *26*(2), 68–75.

Warnakulasuriya, S. (2009). Global epidemiology of oral and oropharyngeal cancer. *Oral Oncology*, *45*(4–5), 309–316.

Wright, A., & Shear, M. (1985). Epithelial dysplasia immediately adjacent to oral squamous cell carcinoma. *Journal of Oral Pathology*, *14*(7), 559–564.

Yang, H. L., Chang, W. H., Chia, Y. C., Huang, C. J., Lu, F. J., Hsu, H. K., et al. (2006). Toona sinensis extracts induces apoptosis via reactive oxygen species in human premyelocytic leukemia cells. *Food and Chemical Toxicology*, *44*(12), 1978–1988.

Yang, C. J., Huang, Y. J., Wang, C. Y., Wang, P. H., Hsu, H. K., Tsai, M. J., et al. (2010). Antiproliferative effect of *Toona sinensis* leaf extract on non-small-cell lung cancer. *Translational Research*, *155*(6), 305–314.

van Oijen, M. G., & Slootweg, P. J. (2000). Oral field cancerization: carcinogen-induced independent events or micrometastatic deposits? *Cancer Epidemiology, Biomarkers and Prevention*, *9*(3), 249–256.