RESEARCH ARTICLE

Tetrahydrocurcumin is more effective than curcumin in preventing azoxymethane-induced colon carcinogenesis

Ching-Shu Lai[1], Jia-Ching Wu[1]*, Shih-Feng Yu[1], Vladimir Badmaev[2], Kalyanam Nagabhushanam[3], Chi-Tang Ho[4] and Min-Hsiung Pan[1]*

[1] Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung, Taiwan
[2] Laboratory of Applied Pharmacology, Staten Island, NY, USA
[3] Sabinsa Corporation, East Windsor, NJ, USA
[4] Department of Food Science, Rutgers University, New Brunswick, NJ, USA

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Scope: Tetrahydrocurcumin (THC), a major metabolite of curcumin (CUR), has been demonstrated to be anti-cancerogenic and anti-angiogenic and prevents type II diabetes. In this present study, we investigated the chemopreventive effects and underlying molecular mechanisms of dietary administration of CUR and THC in azoxymethane (AOM)-induced colon carcinogenesis in mice.

Methods and results: All mice were sacrificed at 6 and 23 wk, and colonic tissue was collected and examined. We found that dietary administration of both CUR and THC could reduce aberrant crypt foci and polyps formation, while THC showed a better inhibitory effect than CUR. At the molecular level, results from Western blot analysis and immunohistochemistry staining showed that dietary CUR and THC exhibited anti-inflammatory activity by decreasing the levels of inducible NOS and COX-2 through downregulation of ERK1/2 activation. In addition, both dietary CUR and THC significantly decreased AOM-induced Wnt-1 and β-catenin protein expression, as well as the phosphorylation of GSK-3β in colonic tissue. Moreover, dietary feeding with CUR and THC markedly reduced the protein level of connexin-43, an important molecule of gap junctions, indicating that both CUR and THC might interfer with the intercellular communication of crypt cells.

Conclusion: Taken together, these results demonstrated for the first time the in vivo chemopreventive efficacy and molecular mechanisms of dietary THC against AOM-induced colonic tumorigenesis.

Keywords:
Aberrant crypt foci / Anti-inflammation / Azoxymethane / Curcumin / Tetrahydrocurcumin

1 Introduction

Curcumin (diferuloylmethane, CUR), a dietary pigment from *Curcuma longa* L., is known to have diverse pharmacologic activities including anti-cancer, anti-inflammatory, anti-oxidant and anti-carcinogenesis [1, 2]. The anti-carcinogenic effects of this compound are demonstrated by its ability to inhibit tumor initiation by azoxymethane (AOM),

Correspondence: Dr. Min-Hsiung Pan, Department of Seafood Science, National Kaohsiung Marine University, No.142, Haijhuan Rd., Nanzih District, Kaohsiung 81143, Taiwan
E-mail: mhpan@mail.nkmu.edu.tw
Fax: +886-7-361-1261

Abbreviations: ACF, aberrant crypt foci; **AOM,** azoxymethane; **COX-2,** cycoloxygenase-2; **CUR,** curcumin; **Cx-43,** connexin-43; **ERK,** extracellular signal-regulated kinase; **GJ,** gap junction; **GJIC,** gap junctional intercellular communication; **GSK-3β,** glycogen synthase kinase-3β; **IHC,** immunohistochemical analysis; **iNOS,** inducible nitric oxide synthase; **PCNA,** proliferating cell nuclear antigen; **THC,** tetrahydrocurcumin

*These authors contributed equally to this work.

benzpyrene and 7,12-dimethylbenz[*a*]anthracene, and to suppress tumor promotion by phorbol esters. Although the anti-inflammatory and anti-carcinogenic activities of CUR have been studied, the action mechanism of CUR is complicated. It is known that CUR administered in mice is reduced to dihydrocurcumin, tetrahydrocurcumin (THC) and trace hexahydrocurcumin by endogenous reductase system in a stepwise manner and subsequently glucuronidated by uridine 5′-diphospho (UDP)-glucuronosyl transferase [3]. THC has been reported exhibited stronger anti-oxidative activities than CUR in several in vitro systems [4, 5]. However, THC was less active in inhibiting 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion than CUR in mouse skin [6]. In contrast to the case of skin carcinogenesis, feeding 0.5% THC in the diet showed stronger inhibition on the 1,2-dimethylhydrazine-induced mouse colon carcinogenesis as compared with CUR [7]. These conflicting findings prompted us to determine the efficacies of CUR and THC to inhibit AOM-induced colon tumorigenesis. Thus, THC was thought to be one of the metabolites with higher physiological and pharmacological activities than CUR in the intestine.

The tumorigenesis of colorectal cancer (CRC) involves various genetic and molecular changes in cell proliferation, inflammation, resistance to apoptosis and tumor angiogenesis [8, 9]. Progression of this disease is commonly characterized by histologically distinct steps, i.e. colonic crypt hyperplasia, dysplasia, adenoma, adenocarcinoma and distant metastasis [10]. During this progression, formation of aberrant crypt foci (ACF) in early stage is believed to be a histological biomarker of colonic tumor development [11]. Mutation and constitute activation of β-catenin and K-ras lead to activation of Wnt/β-catenin/Tcf4 signaling pathway, which subsequently causes the transcription of downstream genes such as myc, cyclin D1, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) that are involved in tumorigenesis [12, 13]. Oncogenic mutation of -*ras* also results in activation of Ras and its downstream effectors, such as Raf/MEK/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)/Akt pathways [14]. Inactivation of glycogen synthase kinase (GSK-3β) through phosphorylation by Akt caused stabilization of β-catenin and its nuclear accumulation [15]. Connexin-43 (Cx-43) is a major component of gap junctional intercellular communication (GJIC), which function to maintain cellular homeostasis. Although Cx-43 has been reported to be a tumor suppressor protein mostly in biological research [16], expression of Cx-43 increase in lymph node metastases of breast cancer compared with primary breast tumors [17]. In addition, β-catenin can regulate the expression of Cx-43 [18]. Nevertheless, it is still unclear whether and how connexins could participate in the development of tumor, especially colon tumorigenesis.

It is known that inflammation is causally linked to carcinogenesis and acts as a driving force in premalignant and malignant transformation [19]. Expression of inflammation-associated enzymes such as inducible nitric oxide synthase (iNOS) and cycoloxygenase-2 (COX-2) contributes to colon tumorigenesis by production of nitric oxide (NO) and prostaglandin E2 (PGE2) in AOM-induced rat and mouse colon cancers [20]. Inhibition of these two enzymes shows protective effects against colon tumor development in different animal models, suggesting they are crucial targets for mucosa inflammation and colon tumorigenesis [9].

In the present study, we investigated the effect of dietary CUR and THC on AOM-induced ACF formation in mice. We also explored the potential mechanisms of their anti-colonic carcinogenesis actions such as proliferation, inflammation and possible signaling molecular pathways. Our results showed that dietary administration of THC was more potent chemopreventive agent than CUR for the prevention of colorectal carcinogenesis.

2 Materials and methods

2.1 Reagents

AOM was purchased from Sigma Chemical (St. Louis, MO, USA). Curcumin and THC were obtained from Sabinsa (East Windsor, NJ, USA). The purity of CUR and THC was determined by high-performance liquid chromatography (HPLC) as higher than 99.2%.

2.2 Animals

Male ICR (Institute of Cancer Research) mice at 5 wk of age were purchased from the BioLASCO Experimental Animal Center (Taiwan Co., Taipei, Taiwan). After 1 wk of acclimation, animals were randomly distributed into control and experimental groups. All animals were housed in a controlled atmosphere ($25 \pm 1^{\circ}$C at 50% relative humidity) and with a 12-h light/12-h dark cycle. Animals had free access to food and water at all times. Food cups were replenished with fresh diet every day. All animal experimental protocol used in this study was approved by Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU).

2.3 Experimental procedure

The experimental protocol for this study is shown in Fig. 1A. Briefly, mice were randomly divided into five groups of five animals each. At 6 wk of age, mice in groups 2, 3, 4 and 5 were given AOM at a dose of 5 mg/kg via an i.p. injection twice a week for 2 wk, and the group 1 was received injection of saline. Group 1 was fed with standard AIN-76 diet and composition as described before [21], while groups 2, 3, 4 and 5 were fed diets containing CUR (0.005 and

0.02%) and THC (0.005 and 0.02%), respectively, continued until the end of study. The diet intake of animals was monitored every day. All animals were sacrificed by CO_2 asphyxiation at 6 or 20 wk for evaluation of aberrant crypts or tumors in colonic tissues. The liver, kidneys and spleen were removed and weighed. The entire colons were excised, cut longitudinally, rinsed with PBS and fixed flat between sheets of filter paper with 10% buffered formalin overnight. All fixed colonic tissues were subjected to ACF and tumor number evaluation or for immunohistochemistry study.

2.4 Determination of ACF and tumors

The formalin-fixed colonic tissues were stained in 0.2% methylene blue solution for 10 min and dipped in distilled water. The total number of ACF in each focus was counted under a microscope ($\times 40$) and expressed as mean $\pm$ SE. ACF were classified with the following morphological characteristics: the enlarged and elevated crypts than in normal mucosa and increased pericryptal space and irregular lumen. The ACF location (distance from anus) and size (number of aberrant crypts) were recorded. Larger ACF were defined as ACF with six or more component crypts. The crypt multiplicity of lesions was determined by transforming the diameter (mm) to crypt multiplicity. Diameters were scored with an eyepiece graticule. The lesions containing >32 aberrant crypts or diameters $\geqq$1 mm were defined as tumors and confirmed by histological H&E stain.

2.5 Western blot analysis

For protein analyses, total scraped colon mucosa was homogenized on ice for 15 s with a Polytron tissue homogenizer and lysed in 0.5-mL ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1% NP-40 and 10 mg/mL leupeptin) on ice for 30 min, followed by centrifugation at 10 000 $\times$ *g* for 30 min at 4°C. The samples (50 μg of protein) were mixed with 5 $\times$ sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% SDS, 25 mM EDTA, 20% glycerol and 0.1% bromophenol blue. The mixtures were boiled at 100°C for 5 min, subjected to stacking gel and then resolved by 12% SDS-polyacrylamide minigels at a constant current of 20 mA. Subsequently, electrophoresis was carried out on SDS-polyacrylamide gels. For Western Blot analysis, proteins on the gel were electrotransferred onto a 45-μ immobile membrane (PVDF; Millipore, Bedford, MA, USA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine and 20% methanol. The membranes were blocked with blocking solution (20 mM Tris-HCl, pH 7.4, 0.2% Tween 20, 1% bovine serum albumin and 0.1% sodium azide) and probed overnight at 4°C with primary antibody (diluted 1:1000 in blocking solution). The primary antibodies used were as follows: iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); COX-2 and Cx-43 monoclonal antibodies (Transduction Laboratories, BD Biosciences, Lexington, KY, USA); phospho-ERK1/2 (Thr202/Tyr204), phospho-GSK3β (Ser 9), β-catenin, ERK (extracellular signal-regulated kinase), and GSK3βpolyclonal antibodies (Cell Signaling Technology, Beverly, MA, USA) The membranes were subsequently probed with anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories) and visualized using enhanced chemiluminescence (ECL; Amersham Bioscience, Piscataway, NJ, USA). The densities of the bands were quantitated with a computer densitometer (AlphaImagerTM 2200 System, Alpha Innotech, San Leandro, CA, USA). All the membranes were stripped and reprobed for GAPDH (Santa Cruz Biotechnology) as the loading control.

2.6 Immunohistochemical analysis (IHC)

Three-micrometer sections of colonic mucosa in ACF and polyps segments were incubated with 0.3% hydrogen peroxide (H_2O_2) in PBS to quench the endogenous peroxidase activity. For antigen retrieval, sections were heated in 10 mM citrate buffer (pH 6.0) (Immuno DNA retriever with citrate, BIO SB, Santa Barbara, CA, USA) in microwave oven for 7 min at reaching full power. Sections were then incubated with PCNA (proliferating cell nuclear antigen; Santa Cruz Biotechnology), β-catenin (1:200), COX-2 (1:100 dilute) and Cx-43 (1:50) primary antibody in PBS for 1 h at room temperature. After washing with PBS, the sections were incubated with a biotin-conjugated horseradish peroxidase secondary antibody (1:200). Immunoreactivity was visualized by standard biotin-labeled secondary antibody and streptavidin-biotinperoxidase for 30 min each. 3,3′-diaminobenzidine tetrahydrochloride (0.05%, DAB) was used as the substrate; positive signal was detected as a brown color under a light microscope.

2.7 Statistical analysis

Relative expression values are given as mean $\pm$ SE for the indicated fold of expression in colon mucosa of mice. A one-way Student's *t*-test was used to assess the statistical significance between the AOM and CUR or THC plus AOM-treated groups. A p-value <0.05 was considered statistically significant.

3 Results

3.1 General observation

During the experiment, all mice were monitored to investigate whether CUR and THC feeding caused any adverse effects. As shown in Table 1, the body weight in each group did not differ or show any unhealthy symptoms throughout

Table 1. Effects of dietary CUR and THC on AOM-induced aberrant crypt foci (ACF) formation in ICR mice

Group	No. of mice examined	Body weight (g)	No. of ACF/colon		
			ACF	Large ACF	Total ACF
AOM	5	36.2±0.8	57±5	40±7	97±9
AOM+0.005% CUR	5	36.7±0.5	37±15[a)]	33±7[a)]	70±21[a)]
AOM+0.02% CUR	5	37.5±0.4	23±4[b)]	30±7[a)]	54±9b[b)]
AOM+0.005% THC	5	36.5±1.0	19±8[b)]	21±9[b)]	39±18[b)]
AOM+0.02% THC	5	37.8±0.4	15±6[b)]	18±6[b)]	33±11[b)]

All mice of each group were killed by decapitation at the end of 6 wk. The colons were removed and fixed in 10% buffered formalin. ACF in formalin-fixed colons were identified as crypts with increased methylene blue staining and expanded pericryptal spaces ($n = 5$). The average number of ACF, large ACF (≧ 6 component crypts/focus) and total ACF were expressed as mean±SE per colon.
a) $p < 0.05$ compared with AOM-treated alone.
b) $p < 0.01$ compared with AOM-treated alone.

the study. Furthermore, no significant difference of the mean weights of liver and spleen, and no pathologic alternations were found among the groups (data not shown). The results suggested no noticeable side effect or toxicity caused by dietary CUR and THC treatment.

3.2 Effect of CUR and THC on AOM-induced ACF formation

The efficacy of dietary administration of CUR and THC on inhibiting AOM-induced ACF formation was determined. Colonic ACF were identified and analyzed under a light microscope after methylene blue staining. Representative photomicrographs are shown in Fig. 1B, and results of the total number of ACF per mouse are summarized in Table 1. All mice developed ACF in the colon after AOM treatment. It has been suggested that larger ACF (containing six or more crypts per focus) have higher risk for malignant tumor progression [22]. Compared with the control group with AOM treatment only, dietary THC-treated mice were lower in total number of ACF and large ACF than CUR-treated group. We found that THC notably reduced the number of large ACF to 21±9 and 18±6 at 0.005 or 0.02%, respectively, compared with 40±7 in AOM-treated group ($p < 0.01$).

3.3 Effects of dietary CUR and THC on AOM-induced polyp formation in mice

We further evaluated the anti-colonic tumorigenesis activity of long-term feeding of CUR and THC. Mice were fed CUR and THC for 23 wk, the colonic tissues were collected and tumors identified were examined by H&E staining. Macroscopic views of the colons and polyps were characterized and the results are shown in Fig. 2. Dietary supplementation of THC markedly inhibited polyp formation in AOM-treated mice. Table 2 summarizes the number of ACF per mice and multiplicity and incidence of polyp. These results demonstrated that long-term feeding of CUR and THC did not cause any effects on body weight. In this study, we observed that AOM-treated group showed 100% polyp incidence; the mean number of ACF and polyp in AOM-treated group was 75±7 and 3.7±0.8. Dietary supplementation of CUR and THC reduced the polyp number to 2.3±1.0, 1.9±0.7 and 1.4±1.6, 0.6±0.8, respectively, in the colon 23 wk after AOM treatment. Administration of THC to AOM-treated mice significantly decreased the tumor incidence ($p < 0.01$). The results suggested that dietary consumption of THC may be more effective than CUR in preventing AOM-induced ACF and colonic polyp formation. We subsequently analyzed the levels of proliferative molecules in colonic tumors. The PCNA-labeling index, a marker for cell proliferation, in the AOM-treated mice increased by 5.0-fold over that of control. Interestingly, dietary THC (0.02%) more potently reduced PCNA-labeling indices than CUR (0.02%) (Fig. 3).

3.4 Dietary CUR and THC inhibited AOM-induced iNOS and COX-2

The inflammatory molecules iNOS and COX-2 have been considered involved in colonic carcinogenesis [20]. Therefore, we next investigated the effects of dietary CUR and THC on AOM-induced iNOS and COX-2 expression in mouse colon. As shown in Fig. 4A, compared with CUR, dietary THC resulted in a dramatic reduction of iNOS protein level in colonic mucosa. ERKs is another key signaling molecule involved in inflammatory response and has been reported involved in inflammation-related colon carcinogenesis [23]. Western blot analysis has shown that AOM treatment induced a dramatic increase in the phosphorylation of ERK1/2. However, feeding with 0.02% THC markedly decreased the phosphorylation of ERK1/2 than CUR (0.02%) (Fig. 4B). In addition, as shown in Fig. 5, compared with CUR, dietary THC resulted in a dramatic reduction of COX-2 protein levels in colonic mucosa at

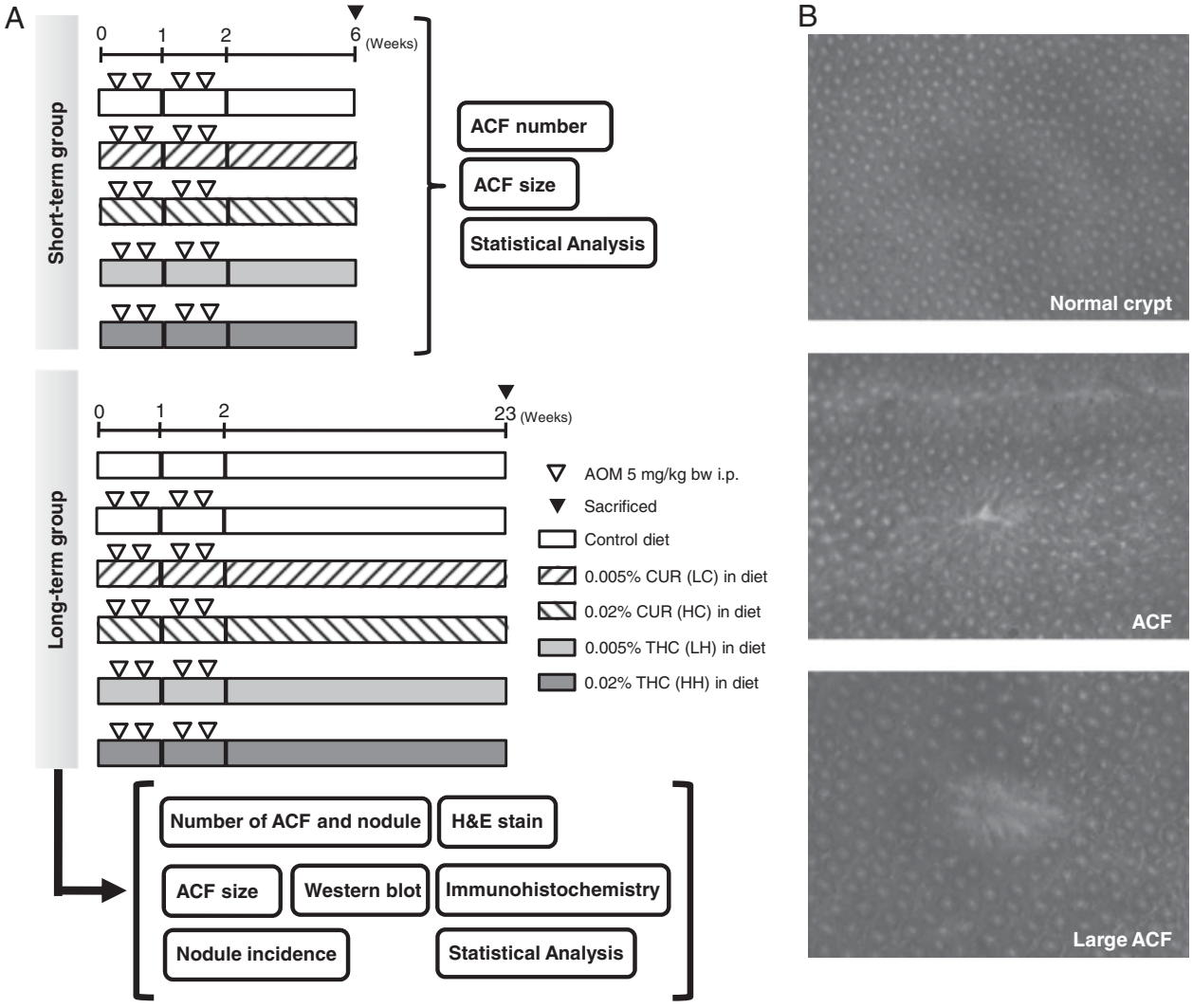

Figure 1. Experimental design (A) and morphology features (B) of AOM-induced colon carcinogenesis in ICR (Institute of Cancer Research) mice. ACF were identified under light microscope (200 × magnification) with methylene blue staining. This revealed a small focus consisting of three (middle) or more than five crypts (large ACF, right).

23 wk after AOM injection. These results suggested that dietary THC treatment may be more potently inhibited inflammatory molecules iNOS and COX-2 expression than CUR in mouse colon possibly through targeting ERK1/2 signaling pathway.

3.5 Dietary CUR and THC modulated AOM-induced β-catenin signaling

β-Catenin is a downstream effector of Wnt/APC/β-catenin signaling pathway that controls colonic epithelial cell proliferation, and commonly dysregulated in colon ACF and tumor [24, 25]. Inhibition of GSK-3β function through phosphorylation by Akt results in cytosolic β-catenin accumulation and subsequent translocates into the nucleus that regulates transcription of oncogenes, such as cyclin D1 and vascular endothelial growth factor [26, 27]. Therefore, we next investigated the regulatory mechanism of THC modulated AOM-induced β-catenin expression. To determine the effect of dietary CUR and THC on β-catenin expression, the colonic tissues were collected and analyzed by IHC and Western blot analysis. As shown in Fig. 6, IHC examination showed that cytoplasmic and nuclear β-catenin intensity was increased when mice were treated with AOM alone as evidenced by dark brown staining. In contrast, dietary THC groups had lighter nuclear staining of β-catenin than CUR in AOM-treated mice. Similarly, Western blotting examination showed that β-catenin intensity was increased in AOM-treated mice compared with

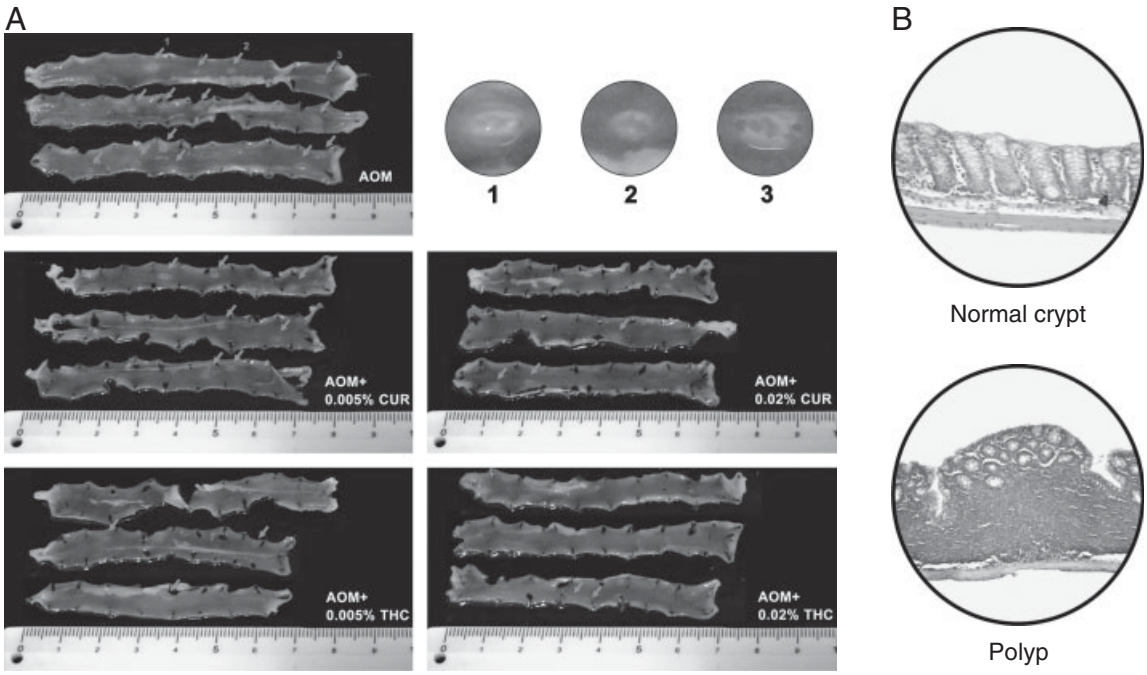

Figure 2. Macroscopic and histologic features of the colon in CUR- or THC-treated mice. (A) Macroscopic views of the colons in each group after 23-wk treatment. The arrows and enlarged photos show (right) the macroscopic polyps. (B) Histopathology of colonic polyps were detected by hematoxylin and eosin stain (200 × magnification).

Table 2. Effects of dietary CUR and THC on AOM-induced aberrant crypt foci (ACF) and polyp formation and in ICR mice

Group	Body weight (g)	No. of ACF/colon			No. of polyp/colon	
		ACF	Large ACF	Total ACF	Polyp multiplicity	Polyp incidence (%)
AOM	41.2±2.1	47±10	28±9	75±7	3.7±0.8	100%
AOM +0.005% CUR	44.6±1.2	33±3[a)]	28±12	61±12[a)]	2.3±1.0[a)]	100%
AOM +0.02% CUR	46.0±0.9	24±6[b)]	17±5[a)]	42±3[b)]	1.9±0.7[a)]	100%
AOM +0.005% THC	44.6±1.6	13±3[b)]	10±3[b)]	23±3[b)]	1.4±1.6[b)]	57%
AOM +0.02% THC	44.4±1.2	5±1[b)]	13±4[b)]	18±5[b)]	0.6±0.8[b)]	43%

All mice of each group were killed by decapitation at the end of 23 wk. The colons were removed and fixed in 10% buffered formalin. ACF in formalin-fixed colons were identified as crypts with increased methylene blue staining and expanded pericryptal spaces ($n = 5$). The average number of ACF, large ACF (≧ 6 component crypts/focus) and total ACF were expressed as mean±SE per colon.
a) $p < 0.05$ compared with AOM-treated alone.
b) $p < 0.01$ compared with AOM-treated alone.

untreated group. THC treatments more lower the level of AOM-induced expression of β-catenin than CUR-treated group (Fig. 7A). Furthermore, we examined the effect of dietary CUR and THC on AOM-induced phosphorylation of GS3K-β As shown in Fig. 8A, in comparison with CUR, dietary THC dramatically suppressed AOM-induced phosphorylation of GS3K-β in a dose-dependent manner (Fig. 8A). Next, we investigated the effect of CUR and THC on AOM-induced Wnt-1, Wnt-3 and Wnt-5a expression. As shown in Fig. 7B, dietary THC inhibited AOM-induced Wnt-1 expression, but this was not found in CUR-treated mice. In contrast, the levels of expression of Wnt-3 and Wnt-5a protein were increased by CUR and THC treatment relative to those in AOM-treated mice. Based on these results, we demonstrated that the decrease in numbers and development of ACFs by dietary THC might be through inhibiting Wnt-1/GS3K-β/β-catenin signaling pathway in AOM-treated mice.

3.6 Inhibitory effects of dietary CUR and THC on AOM-upregulated Cx-43

Recent studies suggested that induction of Wnt1/β-catenin signaling pathway in a mammary epithelial cell line led to an increase in gap-junctional communication and Cx-43

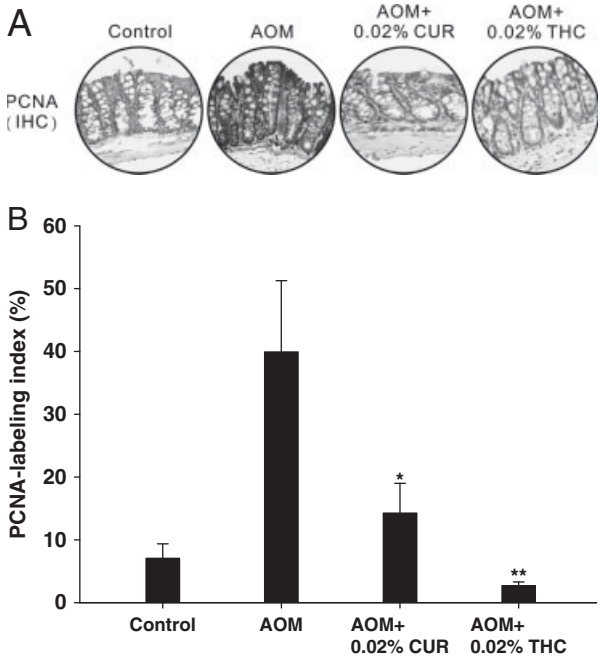

Figure 3. Effects of CUR and THC on AOM-induced proliferation in colorectal tissue. Mice were sacrificed after AOM-injection and fed diet containing CUR and THC for 23 wk. (A) Colonic tissues were embedded in paraffin and visualized by immunohistochemical analysis using PCNA antibody demonstrated as dark stain (200 × magnification). (B) Quantification of PCNA-labeling index (%) was determined in five different fields (× 200) for each mouse by Image J (National Institutes of Health, USA). $^{*}p<0.05$ and $^{**}p<0.01$ indicate statistically significant differences from the control group. All analyses are representative of at lease three independent experiments.

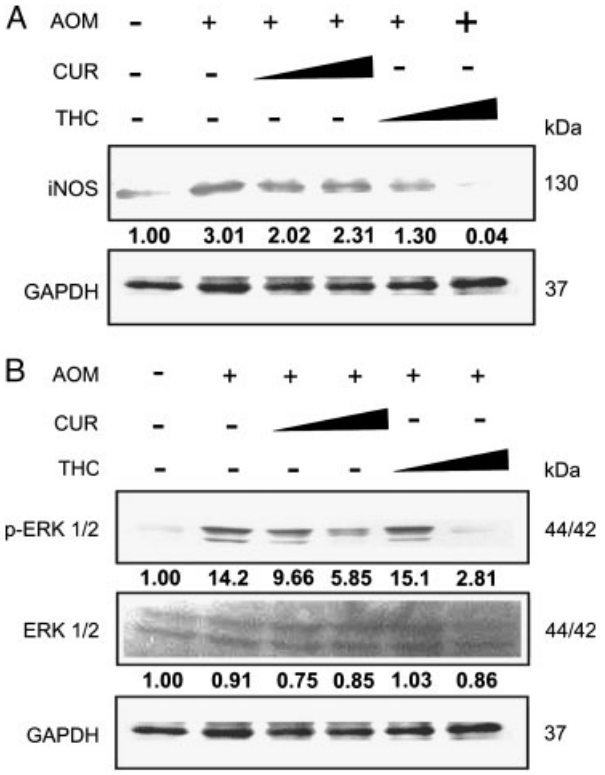

Figure 4. Effect of dietary CUR and THC on AOM-induced inflammatory molecules in colorectal mucosa. Mice were treated as described in Section 2. After 23-wk treatment, colonic mucosa were excised and collected. Total protein lysates from colonic mucosa were prepared and protein levels of iNOS (A) and phosphorylation of ERK1/2 (B) were analyzed by Western Blot. Data are representative of at least three independent experiments, which showed a similar result.

protein expression [28]. To determine the effects of dietary CUR and THC on AOM-induced Cx-43 expression, the colonic tissues were collected and analyzed by Western blot and IHC analysis. As shown in Fig. 8A, compared with CUR, dietary THC markedly inhibited AOM-induced Cx-43 protein expression in a dose-dependent manner. Similarly, IHC examination showed that administration of THC significantly suppressed AOM-induced Cx-43 expression as compared with AOM-alone group (Fig. 8B). These results indicated that dietary CUR and THC suppressed AOM-induced colonic tumorigenesis possibly through inhibiting GJIC mediating intercellular exchange of growth signals by downregulating Cx-43-related control of cellular proliferation.

4 Discussion

The present study was to elucidate the colon tumor-inhibitory activity of THC, a major metabolite of CUR [3]. Previous studies have demonstrated that dietary administration of CUR inhibited AOM-induced colon carcinogenesis in male F344 rats [29]. In this study, for the first time, we compare the chemopreventive effect of CUR and THC in AOM-induced ACF formation in mice. We further elucidate their molecular mechanisms of anti-inflammation and gap junction (GJ) intercellular communication in AOM-induced colonic polyps. The results of this study are in agreement with earlier investigations showing that dietary CUR inhibits AOM-induced colon carcinogenesis. Furthermore, we demonstrated that the chemopreventive effect of THC was more potent than CUR and associated with a decreased inflammation, cell proliferation as well as modulation of the Wnt-1/β-catenin signaling pathways in mice. Long-term (23 wk) dietary consumption of THC also caused reduction of colon polyps multiplicity without any noticeable effects, indicating long-term safety and chemopreventive efficacy of dietary THC. These findings strongly suggested the chemopreventive potential of dietary administration of THC against colonic tumorigenesis.

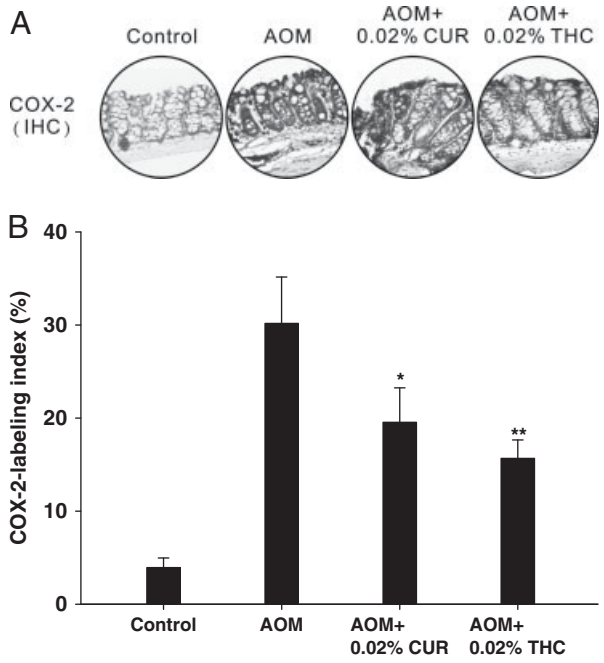

Figure 5. Inhibitory effects of CUR and THC on AOM-induced COX-2 expression in colorectal tissue. Mice were sacrificed after AOM-injected and fed diet containing CUR and THC for 23 wk. (A) Colonic tissues were embedded in paraffin and visualized by immunohistochemical analysis using COX-2 antibody demonstrated as dark stain (200 × magnification). (B) Quantification of COX-2-labeling index (%) was determined in five different fields (× 200) for each mice by ImageJ (National Institutes of Health). $^{*}p<0.05$ and $^{**}p<0.01$ indicate statistically significant differences from the control group. All analyses are representative of at least three independent experiments.

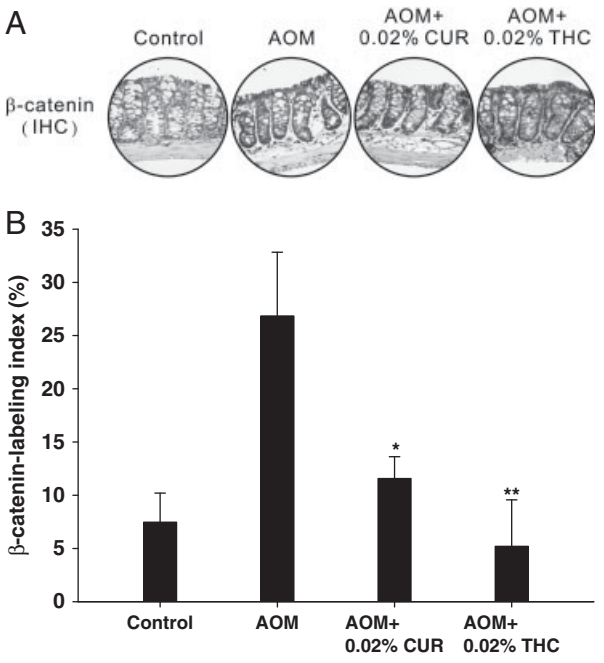

Figure 6. Inhibitory effects of CUR and THC on AOM-induced β-catenin expression in colorectal tissue. Mice were sacrificed after AOM-injection and fed diet containing CUR and THC for 23 wk. (A) Colonic tissues were embedded in paraffin and visualized by immunohistochemical analysis using β-catenin antibody demonstrated as dark stain (200 × magnification). (B) Quantification of β-catenin-labeling index (%) was determined in five different fields (× 200) for each mice by ImageJ (National Institutes of Health). $^{*}p<0.05$ and $^{**}p<0.01$ indicate statistically significant differences from the control group. Data are representative of at least three independent experiments, which showed a similar result.

Overexpression of iNOS and COX-2 enzymes is contributed to promote tumorigenesis by induction of inflammation, abnormal cell proliferation and decreasing apoptosis [30]. In the current study, we showed that dietary THC significantly inhibited AOM-induced increase protein levels of iNOS and COX-2 (Figs. 4 and 5). These results suggested that anti-inflammatory efficacy of THC might be one of the mechanisms for its chemopreventive activity in inhibiting AOM-induced colonic tumorigenesis. Moreover, dietary THC (0.02%) markedly inhibited AOM-induced phosphorylation of ERK1/2, but this was not found in administration of 0.005% THC. However, dietary CUR attenuated the phosphorylation of ERK1/2 in a dose-dependent manner (Fig. 4B). The results suggested that dietary CUR and THC inhibited AOM-induced ACF formation possibly through modulating or interferred different signaling pathways.

Aberrant β-catenin expression and signaling play an important role in colonic tumorigenesis [27]. In our study, we found that THC is more potent than CUR in inhibiting AOM-induced nuclear accumulation of β-catenin through decreasing the phosphorylation of GSK3β; therefore, it reduced the aberrant crypt cell proliferation and ACF formation (Figs. 3 and 6).

In cellular regulation, GJs play multiple roles including GJIC, the formation of hemichannels (HCs) and the use of components such as Cx43 as intracellular signaling transduction factors. GJIC mediating intercellular exchange of growth signals and inflammatory factors in the cytosol or nucleus indicate that connexin-related control of cellular proliferation is important [31]. Moreover, a critical level of Cx43 in the cell is required to maintain adequate proliferation and attenuate the activation of apoptosis [32]. Importantly, we found that the dietary feeding with CUR and THC markedly reduced the protein level of Cx-43 indicating both CUR and THC might interfere with the intercellular communication of crypt cells (Fig. 7). It therefore suggests that dietary CUR and THC suppress the colonic polyps formation possibly through inhibiting GJs formation further blocking cancer cell communication and proliferation.

In summary, this is the first investigation with evidence that dietary THC has great potential as a novel chemopreventive

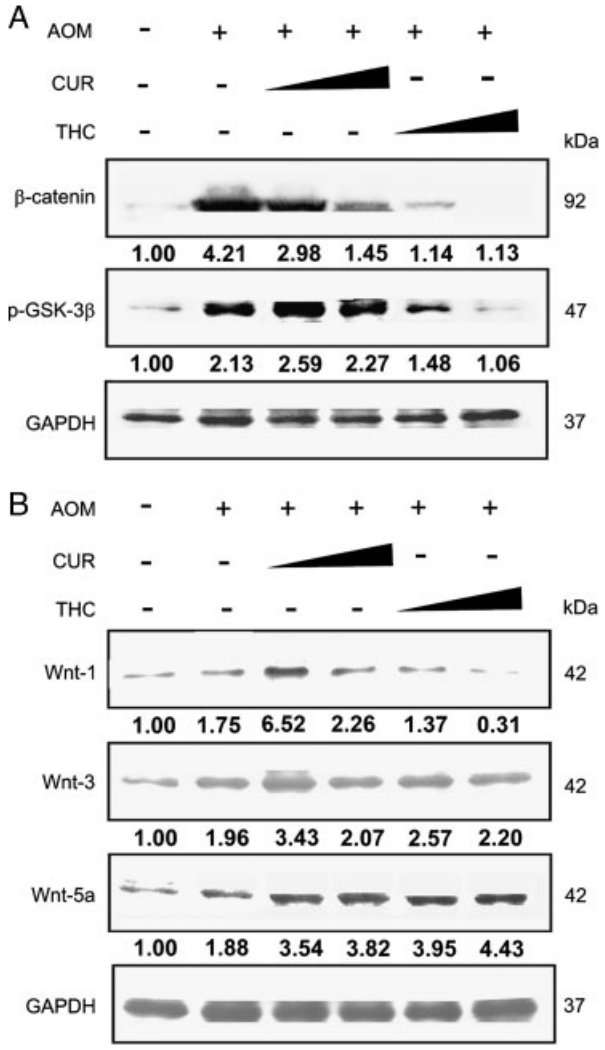

Figure 7. Effect of dietary CUR and THC on AOM-induced Wnt/β-catenin signaling in colorectal mucosa. Mice were treated as described in Section 2. After 23-wk treatment, total protein lysates from colorectal mucosa were extracted and subjected to Western blot analysis for β-catenin and p-GSK-3β (A) and Wnt family proteins (B). Data are representative of three independent experiments.

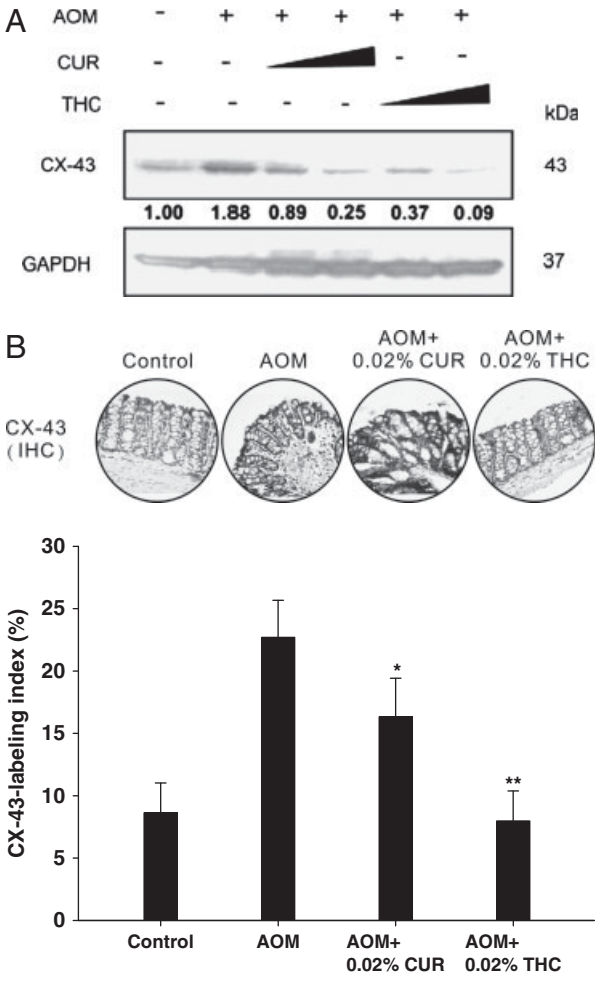

Figure 8. Inhibitory effects of CUR and THC on AOM-induced Cx-43 protein expression in colorectal tissue. Mice were sacrificed after AOM-injection and fed diet containing CUR and THC for 23 wk. (A) After 23-wk treatment, total protein lysates from colorectal mucosa were extracted and subjected to Western blot analysis for connexin-43 (Cx-43), a major protein of gap junction formation. (B) Colonic tissues were embedded in paraffin and visualized by immunohistochemical analysis using Cx-43 antibody demonstrated as dark stain (200 × magnifications). Quantification of Cx-43-labeling index (%) was determined in five different fields (× 200) for each mice by ImageJ (National Institutes of Health). $^{*}p<0.05$ and $^{**}p<0.01$ indicate statistically significant differences from the control group. All analyses are representative of at least three independent experiments.

agent than CUR to be used in the treatment of inflammation associated with tumorigenesis, especially in the prevention and treatment of colorectal cancer. Our results provide evidence for the use of THC supplement as an important chemopreventive agent for colon tumorigenesis.

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The authors have declared no conflict of interest.

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