

# Inhibitory effects of *Curcuma aromatica* oil on proliferation of hepatoma in mice

Wan Yin Wu<sup>1</sup>, Qin Xu<sup>2</sup>, Ling Chun Shi<sup>3</sup> and Wei Bin Zhang<sup>3</sup>

**Subject headings** *Curcuma aromatica*-oil; liver neoplasms; cell proliferation; mice

Wu WY, Xu Q, Shi LC, Zhang WB. Inhibitory effects of *Curcuma aromatica* oil on proliferation of hepatoma in mice. *World J Gastroentero*, 2000;6(2): 216-219

## Abstract

**AIM** To reveal the inhibitory effects of *Curcuma aromatica* oil (CAO) on cell proliferation of hepatoma in mice.

**METHODS** Two tumor inhibitory experiments of CAO on hepatoma in mice were conducted. The inhibitory effects of CAO on proliferation of hepatoma in mice were evaluated by DNA image cytometry and immunohistochemical staining of proliferating cell nuclear antigen (PCNA).

**RESULTS** The tumor inhibitory rates of CAO were 52% and 51% in two experiments, respectively. Compared with those of the saline-treated control groups, both differences were statistically significant ( $P < 0.01$ ). In the group of mice treated with CAO, the cellular nuclear DNA OD value ( $249 \pm 70$ ), are as ( $623 \mu\text{m}^2 \pm 228 \mu\text{m}^2$ ) and DNA ( $2.38 \pm 0.67$ ) index of hepatic carcinomas were significantly lower than those of the control group ( $430 \pm 160$ ,  $1073 \mu\text{m}^2 \pm 101 \mu\text{m}^2$  and  $4.48 \pm 0.71$ ). CAO also could increase diploidy cell rates ( $29.00\% \pm 9.34\%$  vs  $2.97\% \pm 5.69\%$ ,  $P < 0.01$ ) and decrease pentaploidy cell exceeding rate ( $30.04\% \pm 15.10\%$  vs  $70.89\% \pm 14.94\%$ ,  $P < 0.01$ ). In the group of mice treated with CAO, the labeling indexes of proliferating cell nuclear

antigen (PCNA-LI) were  $30\% \pm 4\%$ , which were significantly lower than  $40\% \pm 6\%$  of the control group ( $P < 0.01$ ).

**CONCLUSION** The inhibition of CAO on the growth of hepatoma in mice might be associated with its depression on cellular proliferative activity.

## INTRODUCTION

*Curcuma aromatica* oil (CAO) is a volatile oil extracted from a traditional Chinese herb, *Curcuma aromatica* Salisb, which exerts various medical activities such as promoting blood circulation to remove blood stasis and treating cancers<sup>[1]</sup>. It contains several major anti-tumor active ingredients: elemicin, curcuminol, curdione, etc<sup>[2-5]</sup>. Our previous clinical and experimental studies revealed that CAO infused via hepatic artery had ideal therapeutic effects on both the patients with primary liver cancer and the rats with transplanted hepatoma<sup>[6,7]</sup>. However, the anti-hepatoma mechanisms of CAO remain unresolved. The aim of the current study therefore is to determine the inhibitory effects of CAO on proliferation of hepatoma in mice.

## MATERIALS AND METHODS

### Materials

Ten g/L of CAO injections were prepared by the department of preparations in our hospital (Lot. 97041601). The 5-fluorouracilum (5-FU) was produced by Hebei Pharmaceutical Factory (Tianjin, Lot 970277). EMAIL-100 type automated Image Cytometry (ICM) was purchased from Yiming Company (Guangzhou). *Immunohistochemical* LSAB kit (K0679) and monoclonal antibody PC10 (M0879) to proliferative cell nuclear antigen (PCNA) were purchased from Dakopatts, Glostrup, Denmark. The statistics software package named Medical Statistics of China Medical Encyclopedia was provided by Department of Health Statistics of West China Medical University.

### Animals

Kunming mice, male, weighing 18g-22g, were provided by the Laboratory Animal Center, the First Military Medical University. The ascites

<sup>1</sup>Department of Cancer, <sup>3</sup>Department of Pathology, the Second Affiliated Hospital of Guangzhou University of TCM, Guangzhou 510120, Guangdong Province, China

<sup>2</sup>Department of Pathology, Guangzhou University of TCM, Guangzhou 510405, Guangdong Province, China

Wan Yin Wu, male, born on 1964-08-10 in He County, Anhui Province, graduated from Anhui TCM College as bachelor in 1986, Shanghai TCM University as M.D. in 1992, and Shanghai Medical University as Ph.D. in 1997. Now he is a postdoctoral fellow, associate professor, specializing in prevention and treatment of hepatic neoplasms with integrated Chinese and western medicine, having 28 papers published. National key project of the 9<sup>th</sup> 5-year Plan for Medicine and Health, No. 96-906-07-04 and Guangdong provincial natural scientific grants, No.980663.

**Correspondence to:** Dr. Wan Yin Wu, Department of Cancer, the Second Affiliated Hospital of Guangzhou University of TCM, 111 Dadelu, Guangzhou 510120, Guangdong Province, China  
Tel. 0086-20-81887233 Ext.368  
Email. wwanyin@hotmail.com

Received 1999-06-23 Accepted 1999-12-25

hepatoma carried by mouse was produced by the Cancer Institute of Sun Yat-Sen University of Medical Sciences.

#### **Anti-hepatoma experiments**

Two anti-tumor experiments were performed in the Grade II animal laboratory of our hospital. The hepatoma ascites carried by mouse was collected and was diluted with normal saline (NS) to  $2 \times 10^{10}$  cancer cells/L. The diluted hepatoma cells were put on ice during the implantation, and 0.2mL of hepatoma cells were subcutaneously transplanted to the right axilla in mice. All operations were performed under sterile conditions. After transplanted for 24h, the mice were numbered and randomly allocated into three groups: negative control group, receiving 0.2mL NS, ip; positive control group, given 20mg and 40mg/kg of 5-FU, ip; and treatment groups, given 100mg/kg of CAO, ip. The mice were housed in plastic cages under a 12-hour light/dark cycle and fed with a standard pellet diet and distilled water *ad libitum*. Some of the experimental details are shown in Table 1. At the following day of the termination of treatment, the mice were sacrificed with dislocation of neck. All tumors were peeled off, weighed and the tumor inhibitory rates were calculated. After the second experiment, all tumor specimens were fixed with 100mL/L neutral formalin for less than 48 hours, embedded in paraffin, and cut into 4 $\mu$ m thick series.

#### **DNA image cytometry**

The sections were dewaxed with xylene, rehydrated in decreasing concentrations of ethanol and the DNA staining was ordinarily performed with Foulgen (Figure 1). The cells in the selected visual fields were scanned in super-speed with a television monitor and the DNA histograms were obtained by calculating the integrated optical density with a computer (EMAIL-100). Several visual fields were randomly observed and 50-100 nuclei were detected for each section. The DNA in the normal mouse hepatic cells at G<sub>0</sub>/G<sub>1</sub> phase was employed as the diploid (2C) control. The measurable cellular parameters and paraphrases by ICM were displayed as the follows. DNA optical density (DO) and nuclear area (NA) reflect respectively DNA quantity and nuclei size in hepatoma cells. DNA index (DI) represents the ratio of DNA quantities at G<sub>0</sub>/G<sub>1</sub> phase between hepatoma cells and normal mouse liver cells, 5CER, 2C and 3-5C stand for pentaploidy cell exceeding rate, percentages of diploid cells and of the cells except 5CER and 3C-5C, respectively.

#### **PCNA staining**

Immunohistochemistry with monoclonal antibody against mouse PCNA was performed by the link

streptavidin-biotin peroxidase method (LSAB kit). Briefly, after sections were deparaffinized with xylene and rehydrated through graded alcohol, they were treated with 30mL/L H<sub>2</sub>O<sub>2</sub> for 10 minutes to inactivate endogenous peroxidase activity. Following a 20-minute blocking step with normal horse serum, the sections were sequentially incubated with PC10 antibody diluted 1:100 for 30min, then in anti-rabbit IgG and in avidin-peroxidase complex for 30 minutes each. The immunoreaction was developed using 3',3'-diaminobenzidine in the presence of H<sub>2</sub>O<sub>2</sub> to produce a brown precipitate. The sections were counterstained with hematoxylin. Positive and negative controls were included in each experiment. Specifically, for the latter the primary antibody was substituted with nonspecific rabbit IgG.

#### **PCNA labeling index**

All of the brown nuclei were regarded as positive for PCNA. The PCNA labeling index (PCNA-LI) was determined by observing more than 2000 nuclei in a few areas of the sections, and the percentage of PCNA-labeled nuclei was used for the analysis. To reduce any interobserver bias, the PCNA-LI of all sections was examined by two experienced pathologists who had no knowledge of the experimental grouping. The mean of the two counts of each observer was considered to be the PCNA-LI.

#### **Statistical analysis**

A statistical evaluation was performed using analysis of variance and *t* test. All statistical analyses were considered to be significant at a *P* value of 0.05 or less.

## **RESULTS**

#### **Inhibitory effects of CAO on hepatoma in mice**

The results from the two experiments showed that the tumor inhibitory rates of CAO on hepatoma were 51.85% and 51.2%, respectively (*P*<0.01) (Table 1). Up to the termination of anti-tumor experiment, the body weight in the mice treated with 5-FU tended to decrease, while in the mice treated with CAO, it increased but lighter than that in the negative control group (*P*<0.05).

#### **Effects of CAO on the DNA in the hepatoma cells of mice**

As shown in Table 2, the DO, NA, DI and 5CER in the mice treated with CAO were lower than those in the control mice. CAO could increase the percentage of 2C hepatoma cells and had no significant effect on the percentage of 3.5C cells.

#### **Effects of CAO on the expressions of hepatoma PCNA in mice**

As shown in Table 3 and Figures 2 - 3, the percentages of PCNA positive nuclei in the mice treated with either CAO or 5-FU were statistically lower than those in the negative control mice (*P*<0.01).

**Table 1 Inhibitory effects of CAO on growth of hepatoma in mice**

Experiment	Drugs	Dose (kg <sup>-1</sup> ·d <sup>-1</sup> )	Number	Body weight change (g)	Tumor weight (g)	Tumor inhibitory rate (%)
1	Ns	0.2mL	7	6.90	1.08±0.22	
	5-FU	40mg	7	-2.00	0.35±0.13	67.6 <sup>b</sup>
	CAO	100mg	7	4.35	0.52±0.16	51.8 <sup>b</sup>
2	Ns	0.2mL	10	5.55	1.72±0.45	
	5-FU	20mg	10	-1.85	0.45±0.15	73.8 <sup>b</sup>
	CAO	100mg	10	4.60	0.84±0.31	51.2 <sup>b</sup>

Results are expressed as  $\bar{x}\pm s$ ,  $n=10$ , <sup>b</sup> $P<0.01$ , vs negative control group.

**Table 2 Effects of CAO on the hepatoma DNA parameters detected with ICM in mice**

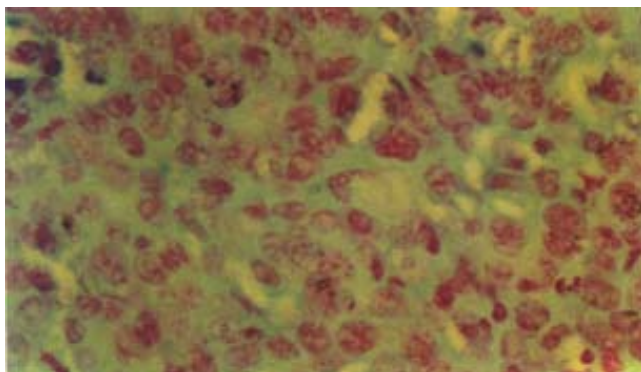
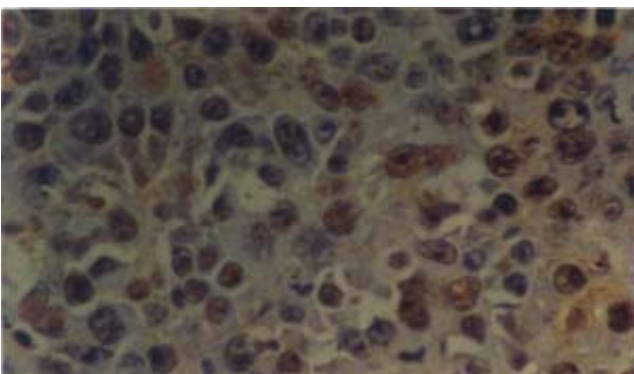
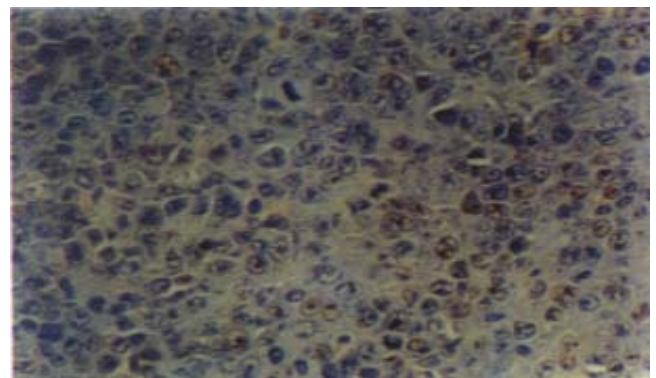
Group	DO	NA(μm <sup>2</sup> )	DI	5CER(%)	2C(%)	3-5C(%)
Negative control	430±160	1073±101	4.48±0.71	70.9±14.9	2.97±5.69	28.2±16.0
CAO	249±70 <sup>b</sup>	623±228 <sup>a</sup>	2.38±0.67 <sup>b</sup>	30.0±15.1 <sup>b</sup>	29.00±9.34 <sup>b</sup>	41.0±17.4

Results are expressed as  $\bar{x}\pm s$ ,  $n=10$ , <sup>a</sup> $P<0.05$ , <sup>b</sup> $P<0.01$ , vs negative control group.

**Table 3 Effects of CAO on the expressions of hepatoma PCNA in mice**

Group	PCNA-LI (%)
Negative control	40.0±5.7
Positive control	25.1±3.6 <sup>b</sup>
CAO	29.9±3.9 <sup>b</sup>

Results are expressed as  $\bar{x}\pm s$ ,  $n=10$ , <sup>b</sup> $P<0.01$ , vs negative control group.

**Figure 1** Feulgen's staining. ×250**Figure 2** PCNA staining for the tumor tissue in the untreated mice, many tumor cells stain positively. ×250**Figure 3** PCNA staining for the tumor tissue in the CAO-treated mice. The positively stained tumor cells were significantly fewer than those of the untreated mice. ×250

## DISCUSSION

The early literature reported that CAO extracted from Chinese herb, *Curcuma aromatica* Salisb, could inhibit the growth of various cancer cells *in vitro* and *in vivo*<sup>[1]</sup>. The recent studies showed that CAO was a compound consisting of many kinds of anti tumor ingredients such as β-elemene, curcuminol, curdione, etc<sup>[2-5]</sup>. However, up to date, few researches on anti-hepatoma mechanisms of CAO compound have been conducted. In our previous clinical study, CAO infused via hepatic artery displayed a remarkable therapeutic effect on the patients with primary liver cancer<sup>[7]</sup>. Our recent animal experimental results revealed that transcatheter infusion of CAO into hepatic artery could significantly inhibit the growth of the implanted hepatoma<sup>[6]</sup>. The present *in vivo* study indicated that the tumor inhibitory rates of CAO were around 50%, which coincided with the results reported by the literature. The changes in body weight of the mice treated with CAO might result from their tumors being smaller than those carried

by the control group mice and the toxicity of CAO being much less than 5-FU.

Some studies have confirmed that ICM used for the DNA quantitative analysis of cancer cells have advantages of briefness, sensitivity and precision as compared with flow cytometry<sup>[8,9]</sup>. In the various parameters obtained by ICM, the DO, NA and DI are positively correlated with cellular proliferation. The percentages of aneuploid cells reflect not only cellular malignancy but also the cellular proliferation. ICM has been employed for analyzing various biological features of cancer cell such as proliferation, differentiation, metastasis and prognosis. Zheng *et al*<sup>[10]</sup> with ICM revealed that the invasiveness and the metastasis of the hepatic tumors enhanced with the increasing DNA synthesis of hepatocellular carcinoma in the patients with liver cancer. Our present study showed that CAO could significantly decrease the DNA quantity (DO, DI) in the hepatoma cells and shrink the nucleus area. In the untreated mice, the higher 5CER and the lower 2C reflected abnormal increasing proliferation. CAO could decrease the percentage of 5CER cells and increase the percentage of 2C, which showed its inhibition of cellular proliferation.

PCNA functions as a cofactor for DNA polymerase  $\delta$  are associated with DNA repair in both the S phase and in DNA synthesis<sup>[11,12]</sup>. Hino *et al*<sup>[13,14]</sup> found that the expressions of PCNA were closely related to the malignancies of human hepatocellular carcinoma. With immunohistochemical methods, Irene *et al*<sup>[15]</sup> and Suehiro *et al*<sup>[16]</sup> affirmed that PCNA-LI was correlated with the metastasis and the prognosis of hepatocellular carcinomas. Therefore, PCNA is an important mark for evaluating the proliferation of hepatocellular carcinomas. In the present study, we used LSAB immunohistochemistry to stain the hepatoma nuclei of mice and the positive PCNA cells were at late G<sub>1</sub> and early S phases. The results indicated that CAO could remarkably decrease the hepatoma PCNA-LI in mice. Therefore, we assume that the inhibition of CAO on the growth of hepatoma in mice might be associated with its depressing PCNA protein, decreasing DNA-polymerase  $\delta$  activity and interfering with DNA synthesis. Combining with the ICM results, we have drawn a preliminary conclusion that the depression of CAO on cellular proliferation could contribute to its inhibition on hepatoma growth in mice. The other possible mechanisms of the inhibitory effects of CAO on hepatomaremains further clarification.

## REFERENCES

- 1 Shi JH, Li CZ, Liu DL. Experimental research on the pharmacology of *Curcuma aromatica* volatile oil. *Zhongyao Tongbao*,1981;6:36-38
- 2 Dong JH, Cheng GB, Hu JH. Isolation and differentiation of  $\gamma$ -elemene from volatile oil of *curcuma wenyujin* and its anti-cancer activity. *Zhongcaoyao*, 1997;28:13-14
- 3 Yang H, Wang XP, Yu LL, Zheng S. The antitumor activity of elemene is associated with apoptosis. *Zhonghua Zhongliu Zazhi*,1996;18:169-172
- 4 Chen LB, Zang J, Wang JH, Hu SY, Zhe XY. Synergism in the cytotoxic effects of  $\beta$ -elemene combined with adriamycin or cisplatin on human gastroadenocarcinoma cell line SGC-7901 *in vitro*. *Zhongliu Fangzhi Yanjiu*,1997; 24:189-191
- 5 Zhou XJ, Gan XS, Wang LX, Qian JM, Li CS, Meng PL. Inhibition of proliferation and induction of apoptosis of elemene on Himeg cell line. *Zhonghua Xueyexue Zazhi*, 1997;18:263-264
- 6 Wu WY, Luo YJ, Cheng JH, Chang G, Liu WS, Li RX. Therapeutic effect of *Curcuma aromatica* oil infused via hepatic artery against transplanted hepatoma in rats. *Huaren Xiaohua Zazhi*,1998;6:859-861
- 7 Cheng JH, Wu WY, Liu WS, Chang G, Liu YL, Yang ZG, Li LN, Zhou H. Treatment of 17 cases of patients with primary liver cancer with *curcuma aromatica* oil infused via hepatic artery. *Shijie Huaren Xiaohua Zazhi*,1999;7:92
- 8 Davey DD, Banks ER, Jennings D, Powell DE. Comparison of nuclear grade and DNA cytometry in breast carcinoma aspirates to histologic grade in excised cancers. *Am J Clin Pathol*,1993;99:708-713
- 9 Schapers RFM, Ploem Zaaier JJ, Pauwels RP, Smeets AWGB, van den Brandt PA, Tanke HJ, Bosman FT. Image cytometric DNA analysis in transitional cell carcinoma of the bladder. *Cancer*,1993;72:182-189
- 10 Zheng YX, Yu YQ, Xu YD, Lin YQ. Correlation between tumor thrombogenesis in portal vein and DNA content in hepatocellular carcinoma. *Zhongliu*,1997;17:145-147
- 11 Lee SH, Hurwitz J. Mechanism of elongation of primed DNA by DNA polymerase  $\delta$ , proliferating cell nuclear antigen, and activator 1. *Proc Natl Acad Sci USA*,1990; 87:5672-5676
- 12 Shivji KK, Kenny MK, Wood RD. Proliferating cell nuclear antigen is required for DNA excision repair. *Cell*,1992;69: 367-374
- 3 Hino N, Higashi T, Nouse K, Nakatsukasa H, Tsuji T. Apoptosis and proliferation of human hepatocellular carcinoma. *Liver*,1996;16:123-129
- 14 Hino N, Higashi T, Nouse K, Nakatsukasa H, Urabe Y, Kinugasa N, Yoshida K, Ashida K, Ohguchi S, Tsuji T. Proliferating cell nuclear antigen and grade of malignancy in small hepatocellular carcinoma evaluation in US-guided specimens. *Hepato Gastroenterology*, 1997;44:245-250
- 15 Irene OLN, Edward CSL, Sheung TF, Matthew N, Annie SYC, Mike KPS. Prognostic significance of proliferating cell nuclear antigen expression in hepatocellular carcinoma. *Cancer*,1994;73:2268-2274
- 16 Suehiro T, Matsumata T, Itasaka H, Yamamoto K, Kawahara N, Sugimachi K. Clinicopathologic features and prognosis of resected hepatocellular carcinomas of varied sizes with special reference to proliferating cell nuclear antigen. *Cancer*, 1995;76:399-405