

## Research Article

# A Novel Antihepatitis Drug, Bicyclol, Prevents Liver Carcinogenesis in Diethylnitrosamine-Initiated and Phenobarbital-Promoted Mice Tumor Model

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Bicyclol, an antihepatitis drug developed by Chinese scientists, has been shown to prevent the malignant transformation induced by 3-methylcholanthrene and 12-*O*-tetradecanoylphorbol-13-acetate in WB-F344 rat liver epithelial cells. This study provides further evidence on its role as a chemopreventive agent in experimental mice with diethylnitrosamine- (DEN-) initiated and phenobarbital- (PB-) promoted liver carcinoma. Liver tissue and serum were collected. In the two-stage model of hepatocarcinogenesis in mice, oral administration of bicyclol (100, 200 mg/kg) before DEN injection showed significant reduction in the incidence of hepatocellular foci, nodules, or carcinoma. Histopathological examination revealed that there was no hepatocellular carcinoma (HCC) and hepatoma formation in the mice pretreated with bicyclol (200 mg/kg) at week 20, while the mice treated with DEN/PB developed 33.3% HCC and 55.6% hepatoma. Furthermore, the serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), and  $\alpha$ -fetal protein (AFP) in serum significantly increased in the DEN/PB model group in comparison with the control group. Pretreatment with bicyclol showed a marked reduction in the above condition. Bicyclol also decreased the expression of AFP and proliferating cell nuclear antigen level in the liver tissue and attenuated the decrease in body weight. In this study, we also found that 10 weeks after stopping the administration of PB and drugs, the control and bicyclol-treated (200 mg/kg) animals showed no HCC and hepatoma formation at the time of termination whereas DEN/PB-induced mice developed 100% hepatoma and 50% HCC. These results further indicate that bicyclol has the chemopreventive potential for liver carcinogenesis induced by carcinogens.

## 1. Introduction

Hepatocellular carcinoma (HCC) comprises 90% of the liver tumors occurring worldwide in humans, with more than half a million new cases reported every year. Diagnosis of this cancer, once restricted mostly to Asia, is now rising in Europe as well as in North America [1]. Environmental factors, such as hepatitis virus infections and chemical carcinogen exposure, are causally implicated in HCC occurrence. About 80% of human HCC cases are attributable to infection [2]. Chronic hepatitis B virus (HBV) carriers are 100–400 times more likely to develop HCC than noncarriers [3]. Hepatitis C virus (HCV) is the second most common cause of HCC

after HBV [4]. Currently, HCC represents more than 4% of all cancer cases in the world and causes at least 315,000 deaths every year [5]. Although early HCC can be cured by surgical resection, many HCC cases are asymptomatic; thus, most HCC patients are not diagnosed in time.

An effective approach to cancer control is chemoprevention, which is expected to interfere with the initiation, promotion, or progression of carcinogenesis. Generally, long-term therapy is required for both chronic HBV and HCV. An antihepatitis drug can be considered to be of great clinical value if it inhibits or suppresses the development of hepatocarcinogenesis besides its improvement of abnormal liver function and inhibition of hepatitis virus replication.

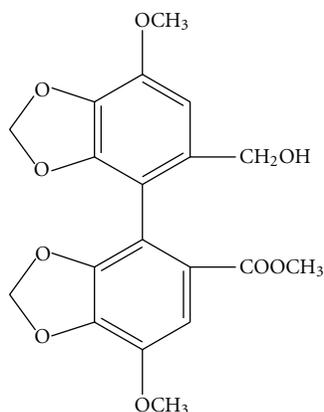


FIGURE 1: The chemical structure of bicyclol.

Bicyclol (4,4'-dimethoxy-2,3,2',3'-dimethylene-dioxy-6-hydroxymethyl-6'-carbonyl-biphenyl) is a novel antihepatitis drug developed by Chinese scientists (Figure 1) [6]. Clinical trials found that bicyclol is effective in improving abnormal liver function and in inhibiting the replication of HBV in chronic hepatitis B patients [7]. Pharmacologically, bicyclol exhibits a protective action against liver injury induced by hepatotoxins in mice and rats, an antifibrotic effect on CCl<sub>4</sub>-induced liver fibrosis in rats and mice, and an antihepatitis virus action in duck viral hepatitis and the 2.2.15 cell line [6, 8, 9]. Furthermore, bicyclol induced differentiation of human hepatocarcinoma cells (HepG<sub>2</sub> and Bel-7402 cells) and reduced AFB<sub>1</sub> hepatotoxicity in rats by increasing the detoxifying metabolism of AFB<sub>1</sub> in rat liver [10]. In the latest study, bicyclol showed a significant inhibitory effect on the malignant transformation of WB-F344 rat liver epithelial cells induced by 3-methylcholanthrene (3MC) and 12-O-tetradecanoylphorbol-13-acetate (TPA) [10]. These results implicate the possibility that bicyclol has a chemopreventive effect on liver carcinogenesis.

The present study aimed to further examine the effect of bicyclol on hepatocarcinogenesis induced by the chemical carcinogens diethylnitrosamine (DEN) as the carcinogenesis initiator and phenobarbital (PB) as the carcinogenesis promoter in mice.

## 2. Materials and Methods

**2.1. Chemicals and Drugs.** Bicyclol, a white crystalline powder with 99% purity, was kindly provided by the Beijing Union Pharmaceutical Plant and was suspended in 0.5% sodium carboxymethylcellulose (Na CMC) for *in vivo* use. DEN, PB, dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), aprotinin, leupeptin, *N,N'*-methylene-bis-acrylamide, and acrylamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) determinations were purchased from Beihuakangtai Chemical Reagent Co., Ltd (Beijing, China). The ELISA kit for  $\alpha$ -fetal protein (AFP) determinations was offered by Shanghai Shenxiong Biotech

Company (Shanghai, China). All the other chemicals used were of analytical grade and were supplied by the Beijing Chemical Agents Company (Beijing, China).

**2.2. Animals.** Male ICR mice weighing 25–27 g (5 weeks old) were purchased from the Beijing Weitonglihua Experimental Animal Co., Ltd (Beijing, China). All mice were bred and maintained under constant conditions at a temperature of 24°C  $\pm$  1°C and humidity of 55%  $\pm$  5% with 12 h light and 12 h dark cycles. Water and feed were accessible to the mice *ad libitum*. Animal care and all experimental procedures were conducted in accordance with the health criteria for care of laboratory animals enacted by the Beijing municipal government.

**2.3. Experimental Protocol.** The experimental mice were randomly divided into 4 groups, and each group contained 15 animals housed at 5 per cage. Liver cancers were induced in all the groups with a single i.p. injection of DEN at a dose of 100 mg/kg body weight in normal saline, except in the control group. After a treatment-free interval of 1 week, the mice were kept on 0.05% PB-containing water whereas the control group was kept on PB-free water for 19 weeks. To examine the prophylactic activities of bicyclol, the animals were pretreated with bicyclol (100, 200 mg/kg) administered orally 2 days before DEN injection. After DEN injection, bicyclol was administered once daily for 6 days/week for a period of 20 weeks.

The mice were weighed periodically and their body weights were recorded. At week 20, 8–10 surviving mice of each group were sacrificed and 5 mice of each group were kept on fresh water and feed for another 10 weeks. Serum was obtained from the blood by centrifugation at 3000 rpm for 10 min. Liver specimens were removed carefully after the mice were sacrificed. Macroscopically visible liver tumors and nodules greater than approximately 1 mm in diameter on the liver surface were recorded. The right lobe of each liver and the nodules were fixed in paraformaldehyde solution for histopathological and immunohistochemical examinations. The remaining liver tissues were stored at –80°C for performing biochemical, ELISA, and Western blot assays.

**2.4. Histopathology.** The fixed liver samples were processed and embedded in paraffin blocks. Tissue block sections were mounted on slides, deparaffinized in xylene, dehydrated in alcohol, and sections with a thickness of 5  $\mu$ m were prepared. Then, the sections were stained with hematoxylin and eosin (H&E). Liver pathological changes and the extent of liver tumors were observed and diagnosed under a microscope by a histopathologist.

**2.5. Biochemical Assays of AST, ALT, and ALP.** The serum levels of AST, ALT, and ALP were measured using commercial kits (Beihuakangtai Chemical Reagent Co., Ltd, Beijing, China).

**2.6. Enzyme-Linked Immunosorbent Assay (ELISA) of AFP.** Quantitative estimation of the tumor marker AFP in liver

and serum followed the corresponding protocols of the kits (Shanghai Shenxiong Biotech Company, Shanghai, China). Serum was obtained from blood by centrifugation at 3000 rpm for 10 min. Liver tissue was homogenized in an electron/grass homogenizer (DY89-I, Ningbo., China) using cold normal saline at 4°C. Then, the homogenates were centrifuged. Aliquots of the supernatants and the serum were used to quantify AFP. The total proteins in liver homogenates were determined by the Lowry methods using bovine serum albumin as the standard. Optical density was measured using scanning full wavelength spectrophotometer (MQX200, BIO-TEK, USA).

**2.7. Western Blot Analysis of PCNA.** The liver tissue was homogenized in an electron/grass homogenizer (Dounce, Ningbo, China) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.3% Triton X-100, 0.03% SDS, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 mM PMSF) at 4°C. After incubation on ice for 30 min, the homogenates were centrifuged at 12000 g for 20 min and the supernatants were boiled in an SDS sample loading buffer for 4 min before electrophoresis on SDS-polyacrylamide gel. After electrophoresis for 2 ~ 3 h, proteins in the SDS-PAGE gel were transferred to nitrocellulose membranes at 27 mA for 12 h at 4°C. The membranes were blocked in 5% milk-Tris buffered saline with Tween 20 (TBST) (20 mM Tris-HCl, pH 7.5, 500 Mm NaCl, 0.1% Tween 20) at 4°C overnight. The blot was probed with a mouse anti-PCNA monoclonal antibody (Santa Cruz Biotechnology Inc., USA) at a dilution of 1 : 200 in 5% milk-TBST for 2 h at room temperature and then incubated with an alkaline-phosphatase-conjugated secondary antibody in skim milk-TBST for 1.5 h at room temperature. The blot was developed using the 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (BCIP/NBT) colorimetric method, and the densities of the bands were determined using the Gel-Pro Analyzer 4.0 software.

**2.8. Immunohistochemistry.** The paraffin-embedded liver tissue was cut into 5 µm thick sections, deparaffinized, and incubated in 3% H<sub>2</sub>O<sub>2</sub> for 10 min to quench endogenous peroxidase activity. After blocking with normal goat serum for 20 min, the sections were stained with a mouse anti-AFP monoclonal antibody (Sigma Chemical Co., USA) and mouse anti-PCNA monoclonal antibody (Santa Cruz Biotechnology Inc., USA) at 4°C overnight, followed by incubation with a horseradish-peroxidase-conjugated goat anti-mouse antibody at 37°C for 30 min. The antibody binding sites were visualized by incubation with diaminobenzidine (DAB)-H<sub>2</sub>O<sub>2</sub> at room temperature for 10 min. Digital images of AFP and PCNA immunoreactive liver cells were acquired at 100x magnification on an Olympus microscope (Olympus, Tokyo, Japan) using an attached Polaroid digital microscope camera (Polaroid, Cambridge, MA, USA) and IBM PC.

**2.9. Statistical Analysis.** The data were presented as the mean ± SD. ANOVA was used for multiple comparisons of groups. The statistical significance between groups was assessed by the paired Student's *t*-test. A value of *P* < 0.05 was considered to be significant.

### 3. Results

The chemopreventive effect of bicyclol on DEN/PB-induced hepatocellular cancer was elucidated in male ICR mice. At week 20, DEN/PB-induced tumors or nodules could be readily observed by unaided eyes (Figure 2(a)). All macroscopically visible liver nodules greater than approximately 1 mm in diameter were counted. Oral administration of bicyclol before tumor initiation resulted in a marked inhibition of tumor development in the animals. The average number of nodules per liver in the bicyclol-treated groups was significantly lower than those in the DEN/PB model group (Figure 2(b)). In addition, the body weight of the animals decreased on DEN/PB administration when compared to the control group. Pretreatment with bicyclol prevented the decrease in body weight (Figure 3).

In the histopathological examination of liver tissue at week 20, the control group animals revealed normal architecture. DEN/PB-induced animals showed loss of architecture and presence of tumor cells. Animals pretreated with bicyclol (100, 200 mg/kg) showed fewer neoplastic cells, near-normal architecture, and significant improvement in liver histopathology (Figure 4). The tumor extent was diagnosed and evaluated by a histopathologist. As shown in Table 1, DEN initiation followed by PB promotion resulted in 33.3% hepatocellular cancer, 55.6% hepatoma, and 88.9% multiple bile canaliculi hyperplasia formations in the liver. Preadministration of bicyclol showed a significant reduction in the incidence of liver tumors. Bicyclol administered at a dose of 200 mg/kg exhibited a stronger preventive effect on liver carcinogenesis as hepatocellular cancer and hepatoma were not observed in the mice at week 20, but 50.0% low-grade multiple bile canaliculi hyperplasia formations were observed.

Table 2 shows the levels of serum marker enzymes: ALT, AST, and ALP. DEN initiation and PB promotion resulted in a marked increase in serum ALT and ALP levels as compared to those in the control group whereas no marked changes were observed in the serum AST levels. Pretreatment with 100 mg/kg bicyclol reduced serum ALT levels significantly. However, bicyclol administered at a dose of 200 mg/kg failed to considerably change the abovementioned levels of ALT, AST, and ALP.

Figure 5(b) depicts the levels of the tumor marker AFP in serum and liver by using the ELISA assay. DEN initiation followed by PB promotion resulted in a marked increase in AFP levels in liver and serum as compared with that of the control group. Pretreatment with bicyclol significantly decreased the AFP level. Bicyclol also reduced the serum AFP level, while there was no statistical significance when compared with the model group because of the large standard deviation. In the immunohistochemistry assay (Figure 5(a)), the AFP

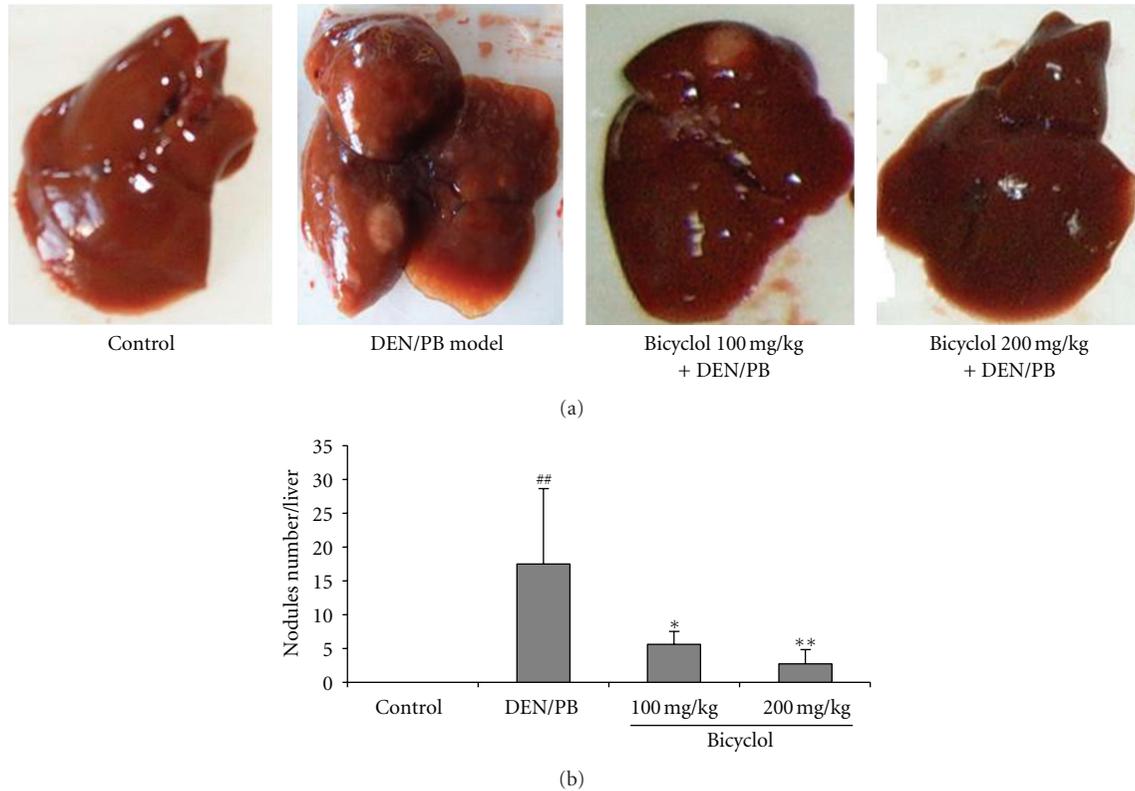


FIGURE 2: Gross liver tumor and nodule phenotypes in DEN/PB-induced ICR mice and the preventive effect of bicyclol at week 20. (a) Photographs of representative livers. (b) Determination of liver tumor and number of nodules per liver greater than approximately 1 mm in diameter. Average values with standard deviations are shown.  $n = 8-10$ . ## $P < 0.01$  compared with the control group; \* $P < 0.01$ , \*\* $P < 0.01$  compared with the DEN/PB model group.

TABLE 1: The incidence of liver tumors induced by DEN/PB in mice and the effect of bicyclol determined by H&E staining and histological evaluation.

Group	Dosage (mg/kg)	HCC	Hepatoma	Cholangioadenoma	Capillary hemangioma	MBCA
Control	—	0.0 (0/6)	0.0 (0/6)	0.0 (0/6)	0.0 (0/6)	0.0 (0/6)
DEN/PB	—	33.3 (3/9)	55.6 (5/9)	22.2(2/9)	11.1 (1/9)	88.9 (8/9)
Bicyclol	100	11.1 (1/9)	22.2 (2/9)	11.1(1/9)	0.0 (0/9)	33.3 (3/9)
	200	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	0.0 (0/8)	50.0 (4/8)

HCC: hepatocellular carcinoma; MBCA: multiple bile canaliculi hyperplasia.

immunoreactivity in Page: 9 DEN/PB-induced liver cells was more intense than that in the control liver cells. Pretreatment with bicyclol markedly decreased the expression of AFP. Using the same ELISA kit, we also found that bicyclol did not exhibit any lowering effect on the physiological elevation of serum AFP in pregnant mice and fetal mice livers treated with bicyclol 200 mg/kg for 10 days (Figure 5(c)).

Figure 6 reveals the immunohistochemical and Western blot analysis of PCNA. In immunohistochemical analysis (Figure 6(a)), the liver sections of the control mice stained for PCNA showed few nuclear-positive cells. However, DEN/PB treatment remarkably increased not only the intensity of immunostaining but also the number of PCNA-positive hepatocytes, which were both significantly reduced with bicyclol

pretreatment. In the Western blot assay, preadministered bicyclol also decreased the increased expression of PCNA induced by DEN/PB (Figure 6(b)).

In this study, we also found that 10 weeks after stopping the administration of PB and drugs, the control and bicyclol-treated (200 mg/kg) animals showed no HCC and hepatoma formation at the time of termination whereas DEN/PB-induced mice developed 100% hepatoma and 50% HCC (data not shown).

#### 4. Discussion

DEN is a potent hepatocarcinogenic nitrosamine present in tobacco smoke, water, cheddar cheese, cured and fried meats,

TABLE 2: Levels of serum marker enzymes ALT, AST, and ALP in all groups.

Group	Dosage (mg/kg)	AST (u/L)	ALT (u/L)	ALP (u/L)
Control	—	332.0 ± 32.8	182.9 ± 25.7	5.3 ± 1.2
DEN/PB	—	334.1 ± 32.1	283.5 ± 55.9 <sup>#</sup>	10.8 ± 6.4 <sup>#</sup>
Bicyclol	100	324.3 ± 19.1	224.4 ± 23.3 <sup>**</sup>	8.4 ± 4.3
	200	350.5 ± 45.6	222.2 ± 53.8	7.8 ± 2.2

Data are means ± SD values ( $n = 8 \sim 10$ ). <sup>#</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ , compared with the control group. <sup>\*\*</sup> $P < 0.01$  compared with the DEN/PB model group.

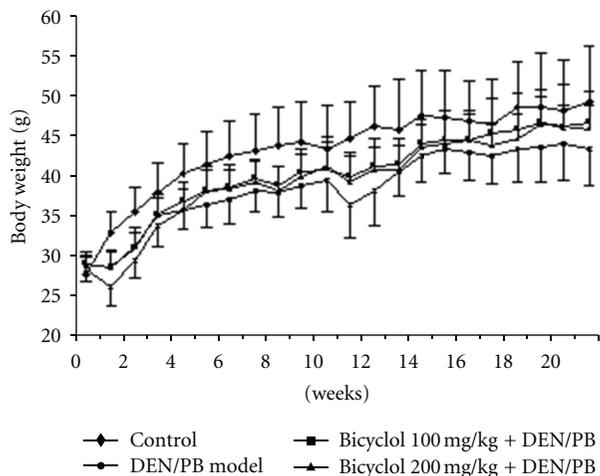


FIGURE 3: Body weight profile in the control and experimental groups. Average values with standard deviations are shown.  $n = 14-15$ .

occupational settings, cosmetics, agricultural chemicals, and pharmaceutical agents. DEN-induced HCC is one of the most accepted and widely used experimental models to study hepatocarcinogenesis [11]. There are 2 liver tumorigenesis protocols for DEN. DEN treatment at the neonatal period (2-week-old mice administered with DEN at a dose of 5 mg/kg body weight) causes severe hepatocyte toxicity. DEN treatment of young adult mice (4-5-week-old mice administered with DEN at a dose of 100 mg/kg body weight) is less toxic and requires tumor promotion by long-term treatment with PB, a procedure referred to as the two-stage protocol [12–14].

In this study, we used the DEN/PB two-stage protocol to determine whether bicyclol has a chemopreventive effect on hepatocarcinogenesis *in vivo*. As female mice are known to be resistant to hepatocarcinogenesis in experimental mouse models, including those employing chemical carcinogenesis, only male mice were used and analyzed in our experiments [15]. In this study, we found that pretreatment with bicyclol (100, 200 mg/kg orally) markedly decreased the number of liver tumor nodules per liver. According to the histopathological diagnosis, the model control group developed 33.3% HCC and 55.6% hepatoma at week 20. However, the mice pretreated with 200 mg/kg bicyclol showed no HCC and

hepatoma formation in their liver. Ten weeks after stopping administration of the promoter and bicyclol, there was still no tumor formation in the 200 mg/kg bicyclol group whereas 100% hepatoma and 50% HCC was observed in the DEN/PB model group. These results further revealed that bicyclol had a preventive effect on hepatocarcinogenesis induced by carcinogens and this preventive activity is persistent.

Liver damage caused by DEN and PB could result in the deterioration or destruction of the cell membrane which leads to the leakage of transaminases and ALP from the liver tissue. The serum levels of transaminases and ALP are representative of the liver function; their increased levels are indicators of liver damage. Cancer chemoprevention and therapy depends on the investigation of these marker enzymes [16]. The elevation of ALT levels is repeatedly credited to hepatocellular damage and is usually accompanied by a rise in AST levels. Increase in ALP levels reflects the pathological alteration in biliary flow. In the present study, pretreatment with bicyclol attenuated the increased activities of ALT and ALP induced by DEN/PB, but only bicyclol- (100 mg/kg) treated mice showed statistically significant results when compared with the DEN/PB group. There were no changes in serum AST levels in the model and bicyclol-treated groups. It is shown that bicyclol helps regenerate the parenchymal cells in liver, protecting membrane integrity and thereby decreasing enzyme leakage (results in [6]). These results shown in this paper also suggest that the protective effect on cell membranes is not the major mechanism by which the chemopreventive effect of bicyclol is exerted.

AFP, an oncofetal serum protein, is progressively lost during development, such that it is virtually absent from a healthy adult [17, 18]. It has been widely used as a clinical marker in the diagnosis and monitoring of HCC. As we previously reported in human hepatocellular carcinoma MHCC97-H cells with high metastatic potential [19], bicyclol pretreatment significantly decreased the production and secretion of AFP. Since AFP overexpression has been associated with uncontrolled growth of HCC [20], our data suggests that bicyclol prevented the progression of HCC that is associated with the downregulation of cell proliferation. Another finding in this study is that bicyclol does not significantly affect the physiological AFP levels in pregnant mice and fetal mice livers. These findings indicate that the lowering effect of bicyclol on AFP is selective. This is an interesting finding, and further studies need to be carried out.

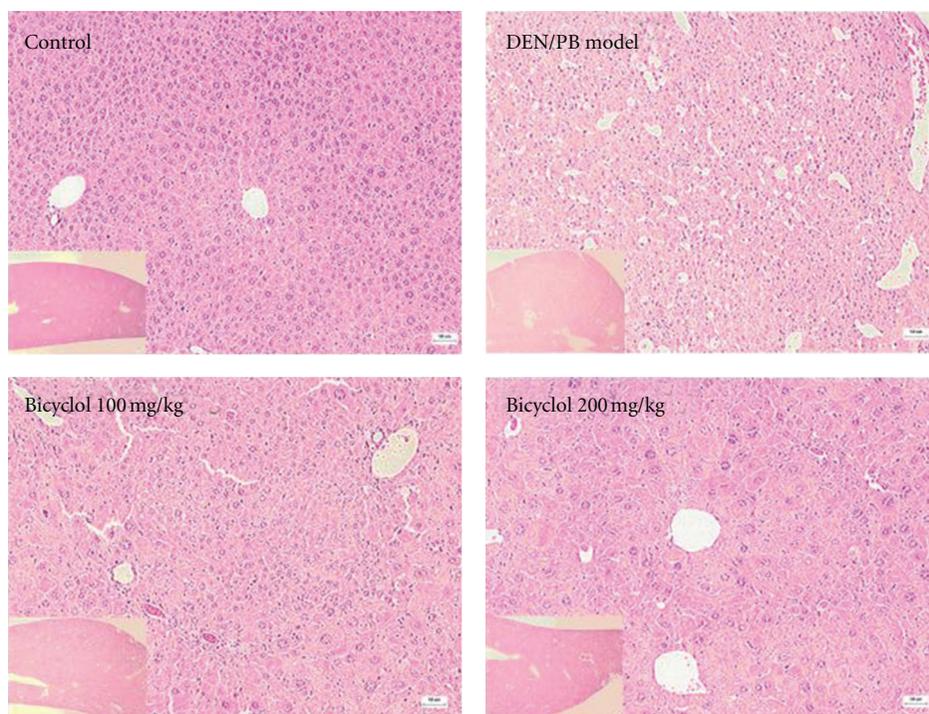


FIGURE 4: Histological study of the liver tissue obtained from the control and experimental groups at week 20. H&E-stained liver sections were photographed at 100x magnification. Insets in the bottom-left corners are images at 20x magnification of the liver tissue from which the 100x photographs were taken. Control group animals revealed normal architecture. DEN/PB-induced animals showed loss of architecture and presence of cancer cells. Animals pretreated with bicyclol (100, 200 mg/kg) showed fewer neoplastic cells and near-normal architecture.

PCNA is another marker of cell proliferation. It plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery [21]. This toroidal-shaped protein encircles the DNA and can slide bidirectionally along the duplex. PCNA has been identified as the DNA polymerase delta accessory protein. In our studies, the expression of PCNA significantly increased in DEN/PB-induced mice in terms of the number of PCNA-positive hepatocytes examined by immunohistochemistry, and the expression of PCNA was quantified by Western blot assay. Pretreatment with bicyclol decreased the expression of PCNA. This data suggests that bicyclol prevents the development of liver tumors along with the inhibition of cell proliferation.

In addition, cytochrome P450 (CYP) monooxygenase also plays a critical role in the activation of various chemical toxicants and precarcinogens [22–24]. Metabolic activation of DEN by CYP enzymes to form reactive electrophiles is required for its toxicity, and PB induces a large spectrum of drug-metabolizing enzymes [25]. According to the results of previous studies [26–28], bicyclol had the modulating activity on CYP isozymes, including the mild inhibition to CYP2C and CYP2D, the mild induction to CYP2E1, and reducing the metabolic rates of the selective substrates of CYP3A, 1A2, 2E1, and 2C. Bicyclol could enhance particularly the denitrosation of DEN, a low toxic pathway of metabolism. Based on these preliminary results, the modulating activity on CYP isozymes of bicyclol may also partly contribute to

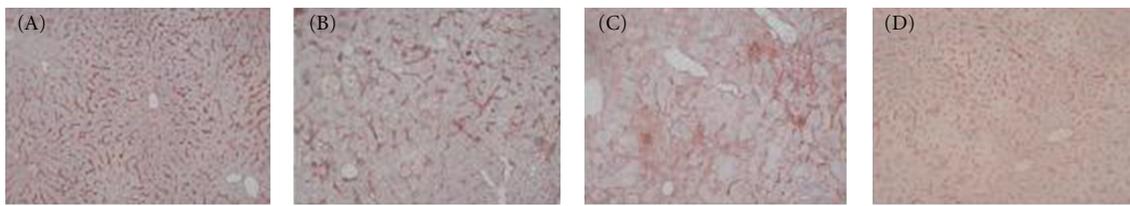
the prevention efficacy on hepatocarcinogenesis induced by DEN and PB found in this paper.

## 5. Conclusions

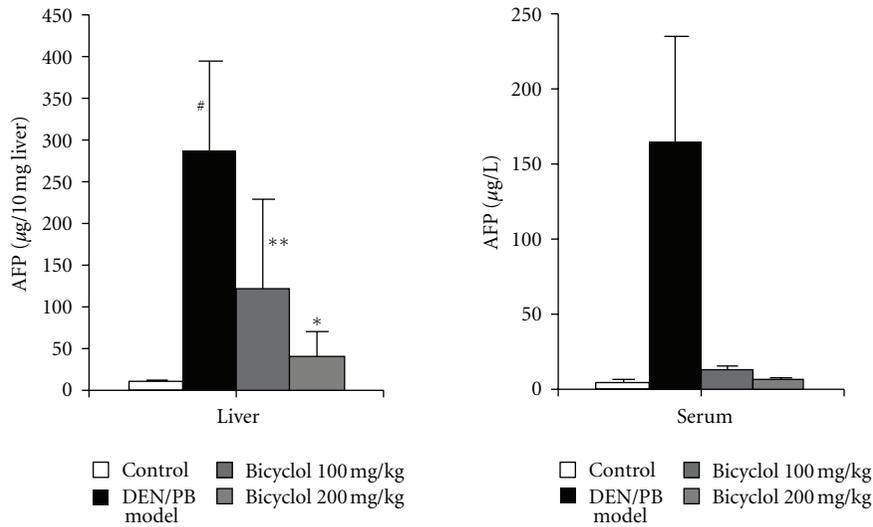
In summary, the findings of the present *in vivo* study are consistent with the results of our *in vitro* studies where we found that bicyclol prevents the malignant transformation of WB-F344 cells induced by 3MC and TPA [10]. Taken together, bicyclol has chemopreventive potential to the liver carcinogenesis induced by carcinogens. This is an interesting finding, and further studies on whether bicyclol can prevent liver carcinogenesis induced by chronic viral hepatitis need to be carried out. Additional studies to understand other molecular mechanisms responsible for its chemopreventive efficacy are warranted to claim bicyclol as a potential chemopreventive agent against liver cancer induced by carcinogens.

## Abbreviations

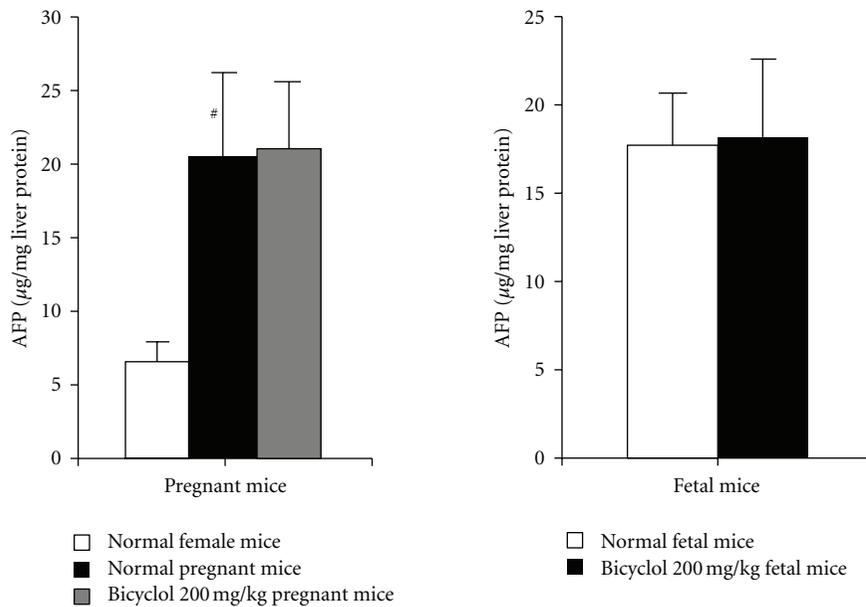
AFP:	$\alpha$ -fetal protein
ALP:	Alkaline phosphatase
ALT:	Alanine aminotransferase
AST:	Aspartate aminotransferase
ELISA:	Enzyme-linked immunosorbent assay
DEN:	Diethylnitrosamine
HBV:	Hepatitis B virus
HCC:	Hepatocellular carcinoma



(a)



(b)



(c)

FIGURE 5: Expression and quantification of  $\alpha$ -fetal protein (AFP). (a) AFP-immunoreactivity assay in the control and experimental groups at week 20 using immunohistochemistry assay. (A) control; (B) DEN/PB model; (C) treatment with bicyclol (100 mg/kg) before DEN injection; (D) treatment with bicyclol (200 mg/kg) before DEN injection. (b) Quantification of serum and tissue AFP levels in the control and experimental groups at week 20 using an enzyme immunoassay kit. Average values with standard deviations are shown.  $n = 8-10$ . <sup>#</sup> $P < 0.05$  compared with the control group; <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$  compared with the DEN/PB model group. (c) Quantification of liver AFP levels in pregnant mice and fetal mice using an enzyme immunoassay kit. Average values with standard deviations are shown.  $n = 4$ . <sup>#</sup> $P < 0.05$  compared with the control group.

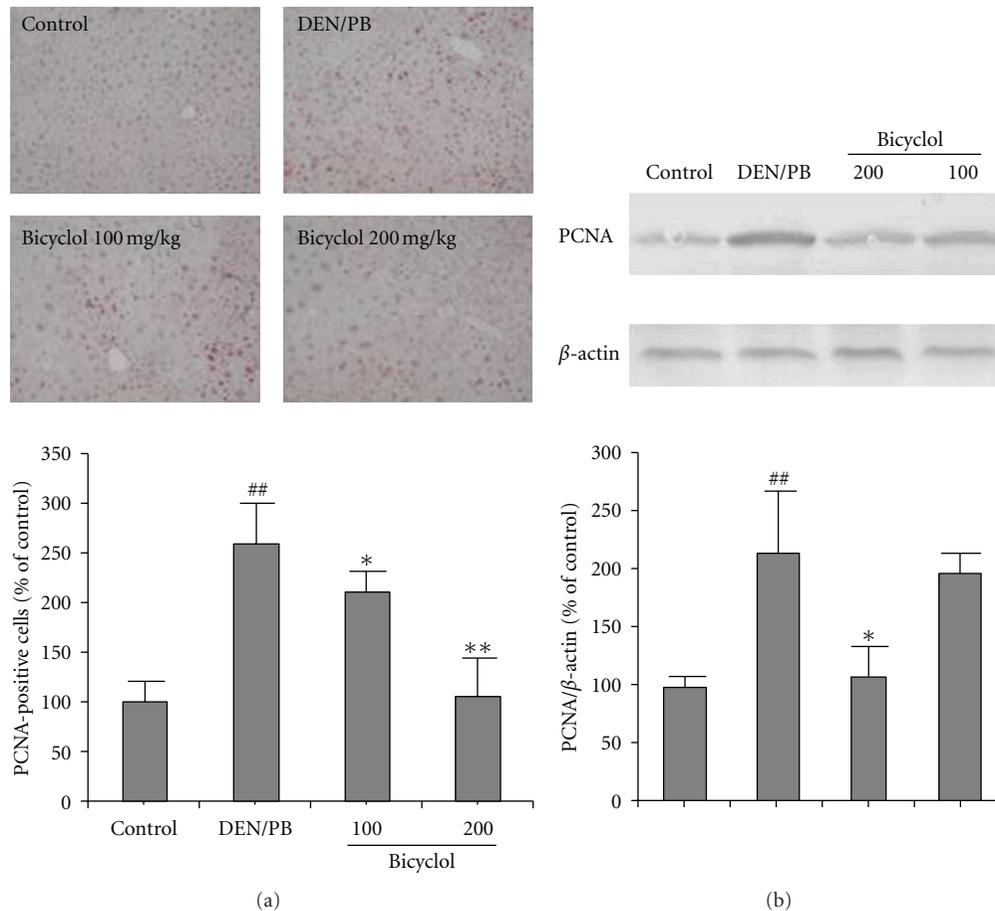


FIGURE 6: Expression of PCNA in the control and experimental groups at week 20. (a) Representative microscopic pictures of PCNA-stained liver sections by immunohistochemical analysis (100 $\times$ ) and quantification of the immunostained section of PCNA. ## $P < 0.01$  compared with the control group; \* $P < 0.05$ , \*\* $P < 0.01$  compared with the DEN/PB model group. (b) Western blot assay of PCNA and the quantitative results obtained by measuring the optical density of each band and expressed as the ratio of each targeted protein and beta-actin. Average values of 3 separate experiments along with the standard deviations are shown. ## $P < 0.01$  compared with the control group; \* $P < 0.05$  compared with the DEN/PB model group.

HCV: Hepatitis C virus

3MC: 3-Methylcholanthrene

PB: Phenobarbital

TPA: 12-*O*-tetradecanoylphorbol-13-acetate.

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