

LIVER CANCER

Effect of Qi-protecting powder (Huqi San) on expression of c-jun, c-fos and c-myc in diethylnitrosamine-mediated hepatocarcinogenesis

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Huqi San can inhibit the over-expression of c-jun, c-fos and c-myc oncogenes and liver preneoplastic lesions induced by DEN.

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Abstract

AIM: To study the inhibitory effect of Huqi San (Qi-protecting powder) on rat prehepatocarcinoma induced by diethylnitrosamine (DEN) by analyzing the mutational activation of c-fos proto-oncogene and over-expression of c-jun and c-myc oncogenes.

METHODS: A Solt-Farber two-step test model of prehepatocarcinoma was induced in rats by DEN and 2-acetylaminofluorene (AAF) to investigate the modifying effects of Huqi San on the expression of c-jun, c-fos and c-myc in DEN-mediated hepatocarcinogenesis. Huqi San was made of eight medicinal herbs containing glycopival granules, in which each milliliter contains 0.38 g crude drugs. γ -glutamyl-transpeptidase-isoenzyme (γ -GTase) was determined with histochemical methods. Level of 8-hydroxydeoxyguanosine (OHdG) formed in liver and c-jun, c-fos and c-myc proto-oncogenes were detected by immunohistochemical methods.

RESULTS: The level of 8-OHdG, a mark of oxidative DNA damage, was significantly decreased in the liver of rats with prehepatocarcinoma induced by DEN who received 8 g/kg body weight or 4 g/kg body weight Huqi San before (1 wk) and after DEN exposure (4 wk). Huqi San-treated rats showed a significant decrease in number of γ -GT positive foci ($P < 0.001$, prevention group: 4.96 ± 0.72 vs 29.46 ± 2.17 ; large dose therapeutic group: 7.53 ± 0.88 vs 29.46 ± 2.17). On the other hand, significant changes in expression of c-jun, c-fos and c-myc were found in Huqi San-treated rats.

CONCLUSION: Activation of c-jun, c-fos and c-myc plays a crucial role in the pathogenesis of liver cancer.

INTRODUCTION

Carcinogenesis is a multi-step process that occurs after a series of discrete, irreversible, and complementary events^[1]. Such genetic damages ultimately lead to either activation of oncogenes (dominant) or inactivation of tumor suppressor genes (recessive). Oncogene is a cancer-causing genetic material (gene) of transformed cells encoding a protein that accelerates and maintains the aberrant growth of the cells.

Over-expression of any kind of proto-oncogenes, such as c-myc, c-jun, c-fos, may also lead to cancer. Over-expression of c-myc is associated with several human cancers^[2,3]. Similarly, over-expression of c-jun and c-fos has also been observed in human cancers^[4,5] and in animals exposed to carcinogens^[6]. Several lines of evidence indicate that some of the known oncogenes in rat liver tissues express under physiological conditions, including pre- and postnatal development, regeneration and neoplasia. Among them, c-jun, c-fos and c-myc oncogenes have been detected in chemically induced rodent hepatocarcinomas. It appears that the expression of some oncogenes may be related to the neoplastic transformation process and their expression products may be used as tumor markers.

However, very little is known about the exact mechanism of such nuclear transcription factors as c-jun, c-fos and c-myc. These nuclear transcription factors are the ultimate targets of growth stimulus. Since c-myc has been identified as the key molecule in various signal

transductions and is permanently activated upon mutation, an attempt should be made to analyze the mutational activation of c-myc proto-oncogene, over-expression of c-jun and c-fos and to correlate these events in DEN and 2-AAF-mediated liver arcinogenesis of Wistar rats.

The initiation of cancer is known as an irreversibly mutational process, which is usually caused by chemical carcinogen, ionizing radiation and virus. Cancer-promoting is a process in which initial hyperplasia cell clone continuously proliferates, and this process is usually reversible. Therefore, it is an ideal target point to prevent cancerization. Huqi San is a Chinese herbal preparation of eight medical herbs, such as *Ramulus Visci*, *Radix Astragali* seu *Hedysari*. Huqi San supplements qi and tonifies the kidney, promotes diuresis, detoxication, and blood circulation. On the other hand, Huqi San has remarkable therapeutic effectiveness against hepatocirrhosis, blocks and reverses hepatocarcinogenesis. Our previous experiment has confirmed that Huqi San has an obvious effect on hepatocarcinogenesis^[7-10]. In this study, a rat model of hepatocarcinogenesis was induced by DEN to observe the function of Huqi San in regulating the expression of c-jun, c-fos and c-myc, and in eradicating hepatocarcinogenesis.

MATERIALS AND METHODS

Animals and treatment

Male Wistar rats weighing 135-149 g (6 wk old) were purchased from the Animal Department of Capital University of Medical Sciences. Seventy rats were allowed free access to pellet diet and water and divided into model group, high dose therapeutic group (8 g Huqi San/kg body weight), small dose therapeutic group (4 g Huqi San/kg body weight), preventive group, part liver group and normal group. The experiment was performed as previously described^[11]. DEN (200 mg/kg body weight) was injected into the abdominal cavity of experimental rats as an initiate agent. After 2 wk, the test entered a selective promoting procedure, which was last for 6 wk. We removed a greater part of liver from rats at the end of the third week. Rats in the treatment group began to take Huqi San for 4 wk after the greater part of liver (PH) was removed, Rats in the preventive group administered Huqi San one week before the model was established, and continued to the end of study. Rats in normal group did not administer Huqi San. After 8 wk, all rats were sacrificed under anesthesia with pentobarbital after 24 h fasting. Hepatic tissue was taken at the right anterior lobe, right back lobe and caudal lobe.

Preparation of Huqi San

Huqi San was made of eight medicinal herbs containing glycoprival granules by gently boiling the herbal drugs, such as *Ramulus Visci*, *Radix Astragali* seu *Hedysari*, *Radix Curcumae* and *Radix Salviae Miltiorrhizae* in distilled water for 60 min to reduce the volume. The herb mixture was soaked for 1 h before boiling. The decoction was then filtered through a delipidated gauze and then concentrated to ointment through decompressing treatment. The

ointment was dried in vacuum to form extractum which was ground. The preparation powder was stored in a refrigerator until use.

Histochemical staining for γ -GTase positive foci

γ -GTase positive foci were determined as previously described^[12]. Fresh liver tissue was cut into 8- μ m thick sections which were mounted on slides and air-dried. Fresh solution containing GMNA, γ -GTase and azo coupling dye, fast blue BBN, 0.1 mol/L tris buffer (pH 7.4) and saline was prepared and then pipetted onto the sections and incubated at room temperature in dark for 30-45 min. Following incubation, the slides were rinsed in saline for 2 min and then transferred to 0.1 mol/L cupric sulfate solution for 2 min. The slides were again rinsed in saline followed by a rinse in distilled water. The sections were air-dried, placed into 10% glycerol and observed under a light microscope. Four sections from each liver were examined under 5 fields. The γ -GTase positive foci counted were divided according to various size ranges and the results were expressed as the number of foci per mm². Sections of kidney tissue served as positive control for γ -GTase staining.

Measurement of 8-OHdG formation in liver DNA

Formation of 8-OHdG formed in liver was detected as described previously^[13]. In brief, DNA was extracted from 500 mg rat liver tissue using a DNA extractor WB kit (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing NaI and OH radical scavenger, with deferoxamine mesylate (Sigma Chemical Co., St. Louis, MO) and RNase (Wako Pure Chemical Industries Ltd., Osaka, Japan). The extracted DNA was digested into nucleosides by combined treatment with nuclease P1 (Yamasa Shoyu Co. Ltd., Chiba, Japan) and alkaline phosphatase (Sigma Chemical Co., St. Louis, MO). Finally the samples were purified using an ultrafree-MC filter unit 100 000 (Millipore Co., Bedford, MA). Formation of 8-OHdG in the resultant samples was quantified by high-performance liquid chromatography (HPLC) as preciously described with certain modification^[14]. Peak gained for 8-OHdG with an electrochemical detector (Shimadzu Corporation, Kyoto, Japan) and dG with an UV detector (MC Medical Inc., Osaka, Japan) was integrated with background noise correction loaded on an integrator. Formation of 8-OHdG was determined by calibration against curves from HPLC runs of standard samples, containing a known amount of authentic 8-OHdG and dG, and expressed as the number of 8-OHdGs per 100 000 dGs.

Immunohistochemical assessment of proto-oncogene expression

The liver tissue was fixed in 10% buffered-formalin solution for 18 h, dehydrated, embedded in paraffin and cut into 6- μ m thick sections. To perform immunocytochemistry, the sections were deparaffinized in xylene, rehydrated in 0.05 mol/L Tris buffer (pH 7.6) for 10 min and boiled in 0.01 mol/L citrate buffer (pH 6.0) for 5 min. The sections were then removed and allowed to cool at room temperature for 20 min and rinsed two times

Table 1 Effect of Huqi San on the number and size of DEN-induced γ -GT positive foci in rat liver (mean \pm SD)

Experimental groups	<i>n</i>	Number of γ -GT foci (per cm ²)	Area of γ -GT foci (mm ² /cm ²)
Normal group	10	0.23 \pm 0.09 ^a	0.18 \pm 0.08 ^a
PH group	12	0.31 \pm 0.08 ^a	0.21 \pm 0.10 ^a
Prevention group	12	4.96 \pm 0.72 ^a	5.12 \pm 3.62 ^a
Large dose therapeutic group (8 g/kg body weight)	12	7.53 \pm 0.88 ^a	8.76 \pm 3.97 ^a
Small dose therapeutic group (4 g/kg body weight)	12	12.77 \pm 0.93 ^b	13.63 \pm 3.44 ^b
Model group	12	29.46 \pm 2.17	21.1 \pm 2.98

^a*P* < 0.001, ^b*P* < 0.01 vs model group; γ -GT: γ -glutamyl-transpeptidase.

with TBS for 30 min. Endogenous peroxidase activity was blocked by incubation for 1 min and 30 s in 3% hydrogen peroxide. To increase antigenic exposure, tissue sections were incubated in 0.1% Triton \times -100 for 45 min at room temperature. The samples were incubated with diluted primary antibodies, rabbit anti-c-fos and c-jun polyclonal antibodies or monoclonal anti-c-myc antibody, for 45 min at room temperature. After rinsed two times with TBS for 20 min, bound primary antibodies were detected by sequential incubation with biotinylated secondary antibody (Biotinylated anti-rabbit or anti-mouse immunoglobulins, LSAB kit from Beijing Zhongshan Biotechnology CO., LTD.) for 30 min, streptavidin peroxidase for 15 min and DAB for 10 min, at room temperature with two rinses in TBS. The sections were then washed with distilled water and counterstained with Mayer's hematoxylin. After dehydration and mounting, the expression of c-jun, c-fos and c-myc in intact rat liver was assessed by immunoperoxidase staining. The negative control for each experiment was a DAKO antibody diluent with background reduction compounds. The positively stained area, brown in color, was determined by Leica Q500 MC image processing and analysis system against the negatively stained region, blue in color^[15].

Statistical analysis

The data were expressed as mean \pm SD and analyzed using SPSS 11.0 software with one-way ANOVA (analysis of variance). *P* < 0.05 was considered statistically significant.

RESULTS

The effect of Huqi San on the number of DEN-induced γ -GTase positive foci in rat liver is listed in Table 1. No γ -GTase positive foci were observed in liver sections from untreated rats (normal group Figure 1A), PH group (Figure 1B) and those from Huqi San-treated group (8 g/kg body weight) (prevention group Figure 1C). γ -GTase positive foci were noted in model rats and the average number of foci was 29.46 (Table 1, Figure 1E). A significant decrease in the number of DEN-induced foci was observed in groups receiving large therapeutic doses of Huqi San (8 g/kg body weight) as compared with model group (Figure 1D). Administration of Huqi San (4 g/kg body weight) after DEN exposure reduced the number of DEN-induced big foci when compared with model group

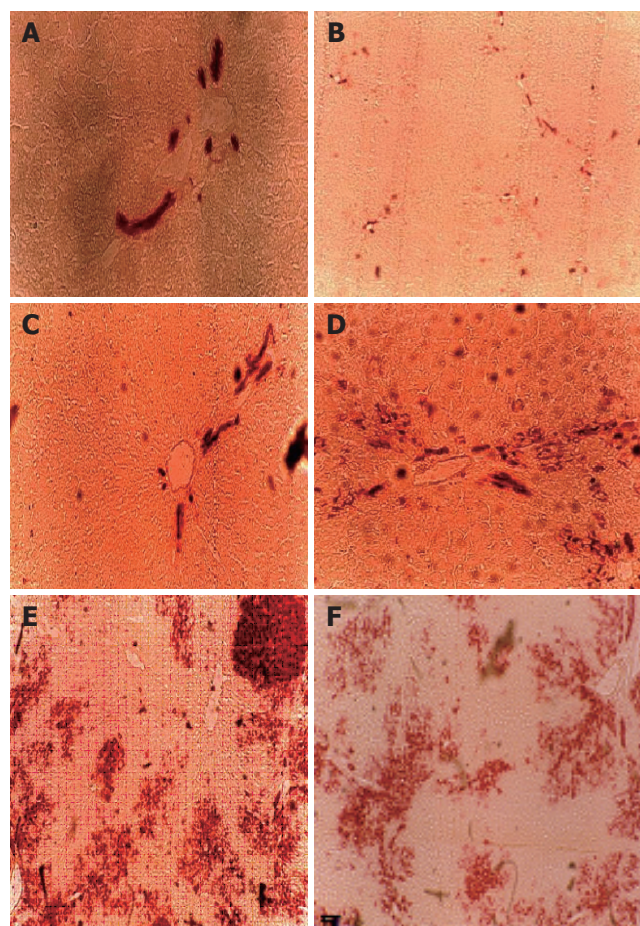


Figure 1 GTase expression in liver sections from the normal control group (A), treated group with PH (removed the greater part liver of rat alone) (B), Huqi San-given group one week before preparation of model rats (C), large dose therapeutic group (8 g/kg body weight) (D), model group (DEN + 2-AAF + PH) (E) and small dose therapeutic group (4 g/kg body weight) (F). The red stained areas are the γ -GTase positive foci with various sizes.

(Figure 1F). Analysis of DEN-induced γ -GTase positive foci before administration of Huqi San or after exposure to DEN for 8 wk showed a decrease in the number of γ -GTase foci (Table 1).

To localize the expression of c-jun, c-fos and c-myc, immuno-cytochemical method was applied to the tissue sections. The expression of c-jun, brown in staining, increased predominantly in model group (3.18-fold of normal control group) in model group (Table 2, Figure 2F). Huqi San (8 g/kg body weight or 4 g/kg body weight) decreased the c-jun expression significantly (*P* < 0.01, Table 2, Figure 2D and E). The expression of c-fos antigen increased 3.14-fold in the model group compared to that in normal control group (*P* < 0.01, Figure 3F) and 1.93- or 2.66-fold in the control group when compared to that in the model group (Table 2, Figure 3D and E). Similar results were observed in c-myc. The expression of c-myc increased 8.25-fold in the model group compared to that in the normal control group (Table 2, Figure 4F) when the dose of Huqi SAN used was 8 g/kg body weight and 4.00- or 5.75-fold when the dose of Huqi SAN used was 4 g/kg body weight (Table 2, Figure 4D and E).

To further investigate the mechanisms involved in the protective action, the effect of Huqi San on DEN

Table 2 Effect of Huqi San on c-jun, c-fos and c-myc protein level in liver (mean \pm SD, mm²)

Experimental groups	c-jun protein		c-fos protein		c-myc protein	
	Level	Fold of control	Level	Fold of control	Level	Fold of control
Normal group	0.28 \pm 0.03 ^a	-	0.29 \pm 0.04 ^a	-	0.12 \pm 0.02 ^a	-
PH group	0.24 \pm 0.02 ^a	0.86	0.29 \pm 0.03 ^a	1.00	0.17 \pm 0.01 ^a	1.41
Prevention group	0.41 \pm 0.01 ^a	1.46	0.33 \pm 0.09 ^a	1.14	0.36 \pm 0.07 ^a	3.00
Large dose herapeutic group (8 g/kg body weight)	0.58 \pm 0.02 ^b	2.07	0.56 \pm 0.02 ^b	1.93	0.48 \pm 0.01 ^a	4.00
Small dose therapeutic group (4 g/kg body weight)	0.71 \pm 0.06 ^b	2.54	0.77 \pm 0.08 ^a	2.66	0.69 \pm 0.06 ^b	5.75
Model group	0.89 \pm 0.03	3.18	0.91 \pm 0.07	3.14	0.99 \pm 0.04	8.25

^a*P* < 0.001, ^b*P* < 0.01 *vs* model group.

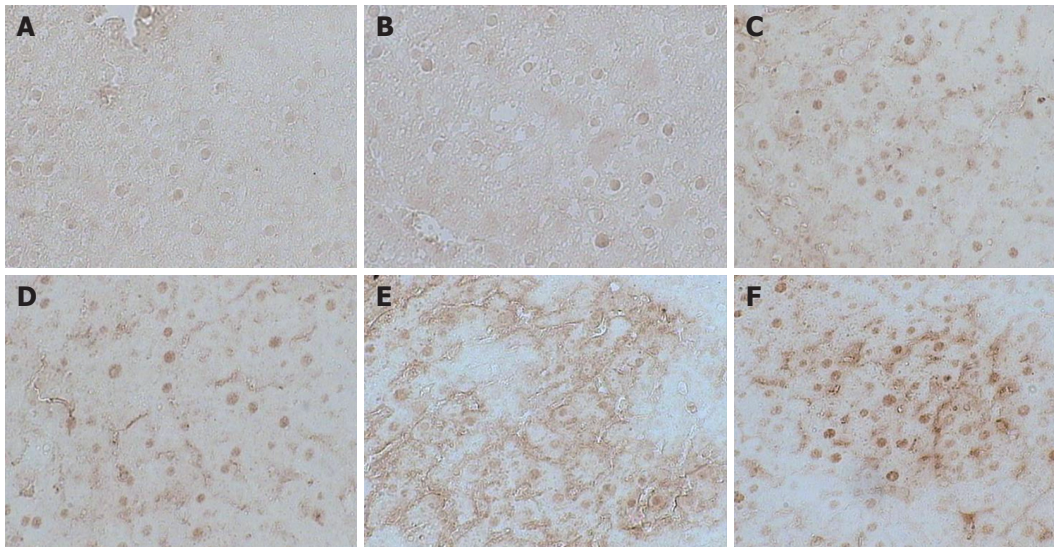


Figure 2 Immunohistochemical examination of c-jun expression in liver sections from the normal control group (A), treated group with PH (removed the greater part of liver from rats alone) (B), Huqi Sangiven one week before the preparation of model rats (C), large dose therapeutic group (8 g/kg body weight) (D), model group (DEN + 2-AAF + PH) (E) and small dose therapeutic group (4 g/kg body weight) (F).

-induced oxidative DNA damage was also determined. The average background level of 8-OHdG found in untreated control rats was $4.06 \pm 0.67/10^5$ dG. Treatment with Huqi San alone did not alter the 8-OHdG level in hepatic nuclear DNA. In DEN-treated rats, 8-OHdG level elevated to $6.5 \pm 1.04/10^5$ dG, which was 60% higher than that in the control group. When rats received Huqi San and DEN concurrently, the 8-OHdG level nearly decreased to that in the control group, indicating that Huqi San was able to protect against DEN -induced oxidative DNA damage.

DISCUSSION

In this study, we assessed the effect of Huqi San on DEN-induced hepatocarcinogenesis using the classical preneoplastic marker enzyme for hepatic chemical carcinogenesis. γ -GTase is a cell surface enzyme initiating the cleavage of extracellular glutathione and glutathione-conjugates. Hydrolysis of glutathione in glomerular filtrate and fluids in other ducts and glands throughout the body provides a mechanism for the body to retain amino acids containing glutathione. In the absence of γ -GTase -initiated cleavage, glutathione is excreted from the body, resulting in fatal cysteine-deficiency. In rodents, γ -GTase is a single copy gene whose expression is regulated in a developmental and tissue specific manner. Several γ -GTase genes or pseudogenes are present in humans. γ -GTase activity is elevated in carcinogen-induced

tumors of animals. Common human epithelial tumors including breast, ovarian and prostate tumors are γ -GTase positive. In human tumor cell lines, induction of γ -GTase and increased intracellular glutathione concentrations have been observed. Synthesis of γ -glutamyl prodrugs is used as a new approach to targeting chemotherapeutic drugs to γ -GTase positive tumors. This placenta form of γ -GTase is hardly detectable in normal rat liver and is markedly induced in liver bearing foci and nodules^[16]. Results from the histochemistry study demonstrated a dramatic protective effect of Huqi San on the formation of γ -GTase positive foci due to DEN exposure (Figure 1). As the appearance of γ -GTase positive foci is correlated with the development of hepatocellular carcinoma, the effect of Huqi San on preventing formation of altered foci and gene expression indicates that Huqi San is capable of protecting against DEN-induced hepatocarcinogenesis.

In recent years, the potential role of oxyradical -induced oxidative DNA damage in carcinogenesis has attracted extensive attention. Oxidative DNA damage refers to all types of structural and functional changes due to the reaction of oxyradicals with DNA, such as base modification and DNA strand breakage^[17]. Among various types of base modifications, 8-OHdG is one of the most important forms of oxidative DNA damage and has been implicated in the development of cancer^[18]. There is experimental evidence that 8-OHdG formation plays an important role in carcinogenesis. Many chemical

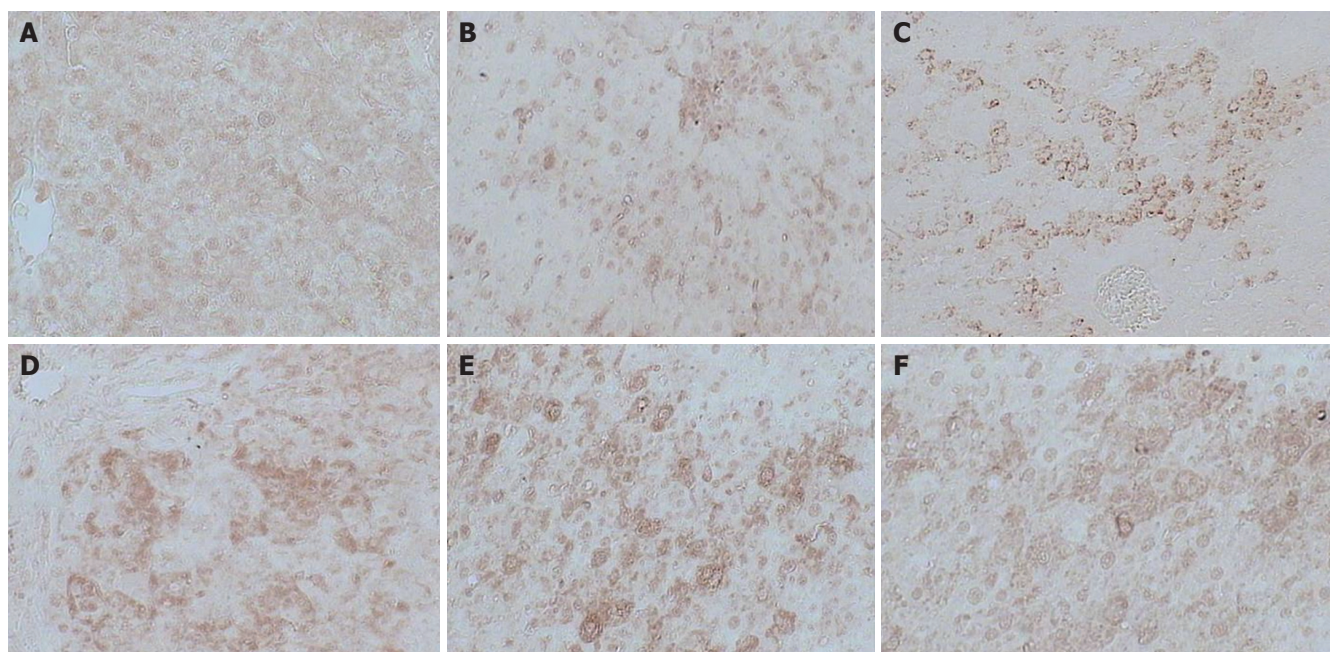


Figure 3 Immunohistochemical examination for c-fos expression in liver sections obtained from the normal control group (A), treated group with PH (removed the greater part of liver from rats alone) (B), Huqi San group given one week before preparation of model rats (C), large dose therapeutic Huqi San group (8 g/ kg body weight) (D), model group (DEN + 2-AAF + PH) (E) and small dose therapeutic Huqi San group (4 g/kg body weight) (F).

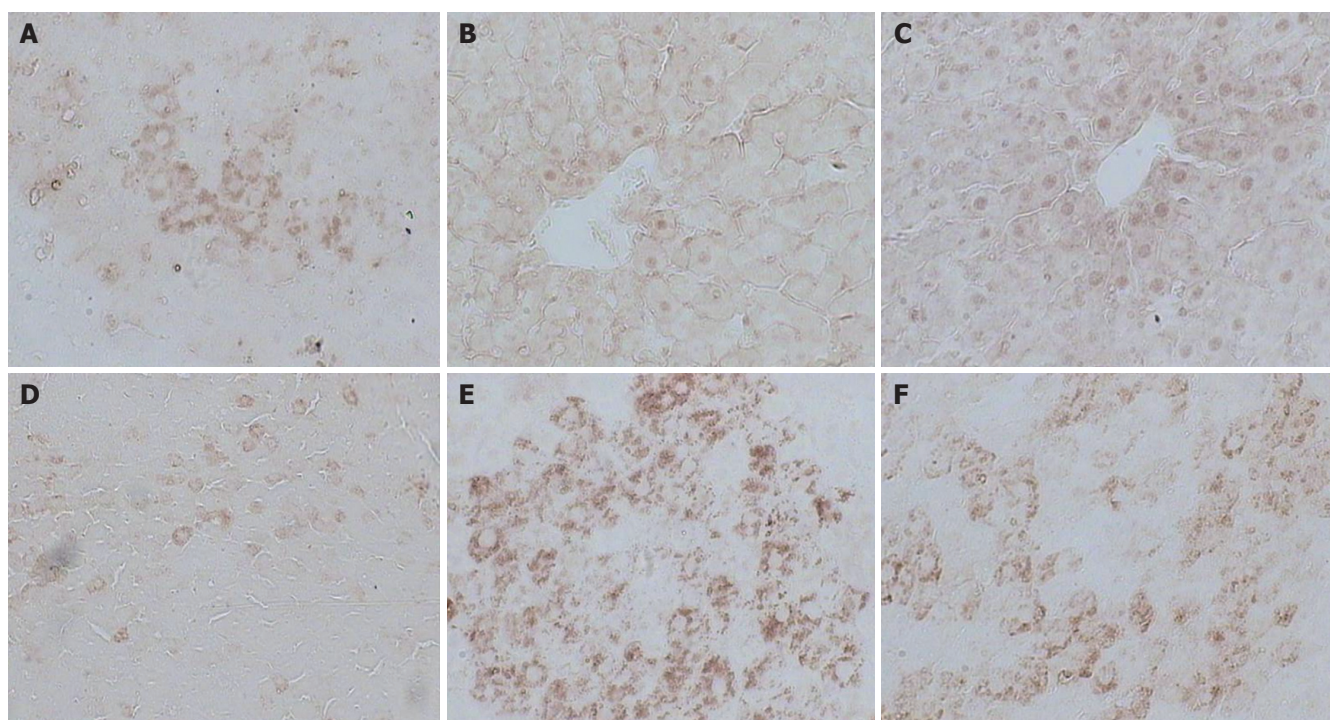


Figure 4 Immunohistochemical examination of c-myc expression in liver sections from the normal control group (A), treated group with PH (removed the greater part of liver from rats alone) (B), Huqi San-given group one week before preparation of model rats (C), large dose therapeutic group (8 g/kg body weight) (D), model group (DEN + 2-AAF + PH) (E) and small dose therapeutic group (4 g/kg body weight) (F).

carcinogens, such as benzo[a]pyrene^[19] and AFB1, DEN^[20,21] can induce 8-OHdG formation in the target organ in a dose- and time-dependent manner. Some chemopreventive agents are found to inhibit 8-OHdG formation caused by carcinogen exposure^[22,23]. The 8-OHdG level in human cancer tissue is significantly

higher than that in corresponding non-tumor tissue^[24,25]. 8-OHdG is associated with the expression of *p53*^[26]. Due to the potential role of 8-OHdG formation in AFB1, DEN carcinogenesis, the inhibitory effect of Huqi San on DEN- induced oxidative DNA damage may have another mechanism underlying its anticarcinogenic effects against

DEN, in addition to reduction of DEN- DNA adduct formation. The formation of 8-OHdG is a result of direct interactions between oxyradicals, especially hydroxyl radicals and DNA bases. Our previous study showed that hydroxyl radicals are involved in initiating oxidative DNA damage caused by DEN^[27]. Therefore, any factor interfering with the generation or action of hydroxyl radicals would affect the formation of 8-OHdG. Results from our previous experiments also showed that Huqi San is able to inhibit DEN -induced oxyradical formation in cultured primary hepatocytes^[28], indicating that by directly scavenging oxygen radicals generated from the metabolic processing of DEN, Huqi San is able to reduce 8-OHdG formation in rat liver.

It was reported that nuclear oncogenes, such as c-jun, c-fos and c-myc could be activated during rat hepatocarcinogenesis with cellular proliferation observed both in regenerating liver and in experimental systems^[29,30]. The expression of c-myc is emphasized as a prognostic indicator for liver carcinoma^[31,32]. In addition, over-expression and localization of c-myc play an important role in initiation, differentiation, and progression of liver carcinomas^[33]. c-jun and c-fos are suggested to be a risk factor for relapse in liver carcinoma^[34], and their expression is suggested to be one of the parameters related with prognosis of liver carcinoma. Furthermore, their expression and activation are presumed to play an important role in the early phase of carcinogenesis of liver carcinoma^[35].

In the present study, c-jun, c-fos and c-myc protein levels were increased in all DEN + 2-AAF + PH-treated animals at early stages. The expression of c-jun, showing a brown staining in the rat liver, increased predominantly in model group (3.18-fold in the normal control group). Huqi San (8 g/kg body weight or 4 g/kg body weight) decreased c-jun expression significantly ($P < 0.01$) and increased 3.14-fold in the model group compared to normal control group. The expression of c-myc increased 8.25-fold in the model group compared to that in the normal control group when the dose of Huqi San was 8 g/kg body weight and decreased 4.00- or 5.75-fold when the dose of Huqi San was 4 g/kg body weight. The effect of Huqi San on preventing formation of altered foci as well as gene expression indicates that Huqi San is capable of protecting against DEN hepatocarcinogenesis.

In summary, Huqi San can be used as a potential anticarcinogenic and antiproliferative agent.

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