

## EFFECT OF LIGUSTRAZINE ON A RAT MODEL WITH HEPATIC PRENEOPLASIA

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### Abstract

Ligustrazine is a natural active alkaloid, widely distributed, with a variety of pharmacological activities. The aim of this study was to assess the effect of ligustrazine on rats with hepatic precancerous status, and to provide evidence for the clinical prevention and treatment of liver diseases, with ligustrazine. For the rat liver cancer model we used the diethyl nitrosamine (DEN) model. 24 male Wistar rats were randomly divided into the control group, DEN group and DEN + ligustrazine group. The rats in DEN group were treated with DEN (55 mg/kg b.w.) i.p. twice a week. The rats in DEN + ligustrazine group were treated with DEN (55 mg/kg b.w.) i.p. twice a week and ligustrazine solution (163 mg/kg b.w.) daily. The rats in the control group were treated i.p. with the same volume of normal saline. After 15 weeks the rats from the three groups were sacrificed and the blood and liver collected for analysis. The liver tissue structure and cell morphology of the three groups of rats were assessed by light microscopy. The expression of alpha-fetoprotein (AFP) in the three groups was verified using immunohistochemistry (IHC). In the DEN group the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutathione S-transferase (GST), total bilirubin (TB), and malondialdehyde (MDA) was significantly increased than in the control group ( $p < 0.01$ ). Compared with DEN group, the levels of AST, ALT, GST and TB in serum in DEN + ligustrazine group was significantly decreased ( $p < 0.05$ ), while MDA in serum was more significantly decreased ( $p < 0.01$ ). The hepatic tissue structure and cell morphology of the rats in the control group were normal. The liver of the rats in DEN model group had hepatic precancerous lesions and the liver cells were characterized by nodular hyperplasia and enhanced shape of typical cancer cells. The bile ducts epithelium was characterized by hyperplasia and significant atypia. The liver of rats in the DEN + ligustrazine group showed the common features of the first three lesions, but did not presented clear cancer cells.

### Rezumat

Ligustrazina este un alcaloid natural cu largă răspândire și cu o varietate de efecte terapeutice. Obiectivul studiului a fost evaluarea efectului ligustrazinei într-un model de șobolan cu preneoplazie hepatică, precum și demonstrarea efectului acesteia în bolile hepatice. Pentru modelul animal de neoplazie hepatică s-a folosit dietil nitrozamina (DEN). 24 de șobolani masculi sușa Wistar au fost împărțiți în lotul martor, lotul DEN și lotul DEN + ligustrazină. Șobolani din lotul DEN au fost tratați cu DEN (55 mg/kg corp) i.p. de două ori pe săptămână. Șobolani din lotul DEN + ligustrazină au fost tratați cu DEN (55 mg/kg corp) i.p. de două ori pe săptămână și soluție de ligustrazină (163 mg/kg corp) o dată pe zi. Șobolani din lotul martor au fost tratați cu același volum de ser fiziologic i.p. ca și loturile de studiu de două ori pe săptămână. După 15 săptămâni șobolani din cele 3 loturi au fost sacrificați iar sângele și ficatul au fost recoltate pentru analiză. Structura și morfologia celulelor țesutului hepatic colectat a fost analizată microscopic. Expresia alfa-feto-proteinei (AFP) în cele trei loturi a fost determinată prin metoda imunohistochimică. În lotul DEN nivelurile aspartataminotransferazei (AST), alanin aminotransferazei (ALT), bilirubinei totale (TB), glutation-S-transferazei (GST), malondialhidei (MDA) au fost semnificativ crescute, comparativ cu lotul martor ( $p < 0,01$ ). În comparație cu lotul DEN, nivelurile serice de AST, ALT, GST și TB la lotul DEN + ligustrazina au fost semnificativ mai scăzute ( $p < 0,05$ ), în timp ce în cazul MDA scăderea este înalt semnificativă ( $p < 0,01$ ). Structura și morfologia celulelor țesutului hepatic la lotul martor au fost normale. Probele din ficatul șobolanilor din lotul modelului DEN au prezentat leziuni precanceroase hepatice, iar celulele hepatice au prezentat hiperplazie nodulară și celule canceroase tipice. Epiteliul canalului biliar a prezentat hiperplazie și atipie semnificativă. Ficatul șobolanilor din lotul DEN + ligustrazină a avut modificări specifice primelor trei leziuni, dar nu și celule canceroase clare.

**Keywords:** ligustrazine, liver cancer, animal models, MDA, alpha-fetoprotein (AFP), HCC

### Introduction

Liver cancer is a very common malignant tumour, and the mortality rate ranks it as the third in the global malignancy. Several risk factors have been identified

to this malignancy as chronic infections with hepatitis B virus or hepatitis C virus, cirrhosis, non-alcoholic fatty liver disease, primary biliary cirrhosis, inherited metabolic diseases, heavy alcohol use, toxins and drugs [7, 9]. Some types of cancers like gastric or colorectal

adenocarcinomas determine secondary liver damages, that could be diagnosed through histochemical and immunohistochemical examinations [8, 23, 30].

Considering the improvement of medical technology, especially the development of medical imaging most of the liver cancers can be diagnosed early and correctly. Currently, there are many ways to treat liver cancer, including surgical resection, liver transplantation, biological therapy and systemic chemotherapy. But all these treatments, especially chemotherapy, are accompanied by severe immunosuppression that can cause severe skin (refractory acne) or lung infections (parapneumonic pleurisy) with bacteria or fungus from *Aspergillum* or *Fusarium* genus that represent a challenge for the clinical microbiology [5, 13, 26].

The first choice of treatment still remains the surgical resection. However, for patients with liver cancer, the recurrence rate express high ranks after surgical resection, and the long-term efficacy is not clear. Many natural extracts [2, 11, 17, 21] have shown anticancer activities, that could determine a new direction in cancer prevention and therapy, so the focus should be on the discovery of new natural products or molecules with cytotoxic properties on different cancer cell lines and to use nanotechnology to design targeted drugs for tumour cell for an effective anticancer treatment [18, 20]. Therefore, researches regarding the prevention of liver cancer are even more important. Nowadays, the main animal models of rat liver cancer are: portability liver cancer model (the tumour strain was injected directly into the experimental animals and cultured to form solid tumours); transgenic animal liver cancer model; induced animal liver cancer model [4]. In this paper, based on the experimental requirements and laboratory conditions, we used the diethyl nitrosamine (DEN) as a tumour-inducing agent. DEN is a chemical substance with significant hepatotoxicity, genotoxicity and immunotoxicity, which belongs to nitrosamines group and it's currently the most widely used drug for inducing liver cancer [15]. DEN is metabolized by the liver microsomal membrane-bound enzyme cytochrome P-450IIE1 to acetaldehyde that causes damage to liver cells by methylation of the nucleic acids and proteins, leading to carcinogenesis and necrosis. Generally, the whole process is divided into several stages, as follows: liver cells begin to damage → hepatocellular hyperplasia → hepatocellular nodular hyperplasia → hepatocellular carcinoma. This model is closer to the clinical characteristics and to the human liver cancer development. So, this experimental model may help to a better understanding of the aetiology and carcinogenesis processes of liver cancer.

*Ligusticum wallichii* (*Umbelliferae*) is a traditional Chinese medicine plant, perennial, with strong rhizome

and a potent aroma [29]. Ligustrazine is a natural active alkaloid, widely distributed in nature, and it mostly exists in a variety of plants in the form of ligustrazine hydrochloride and ligustrazine phosphate. It is the effective chemical constituent of *Ligusticum wallichii*. Modern studies have shown that ligustrazine has a variety of pharmacological activities, such as anti-tumour, anti-tissue fibrosis, prevention and treatment of cardiovascular and cerebrovascular diseases, vasodilatation, improvement of microcirculation [6, 27]. Many researchers have studied the mechanism of action of ligustrazine in some human liver pathologies. For example, Feng L. *et al.* [10] studied the protective effect of ligustrazine on renal ischemia/reperfusion injury; Wang [28] studied the pharmacokinetic features of ligustrazine in mice blood, brain and liver. Lu [16] studied the preventive role of ligustrazine on alcohol-induced liver injury by reducing liver steatosis and oxidative stress. However, the research of ligustrazine for the simple prevention of liver cancer is relatively rare. Taking into account these aspects we investigated the effects of ligustrazine on hepatic precancerous lesions in rats through the DEN model, aiming to provide the basis for future studies for determining the hepatoprotective and anti-cancer action mechanisms of ligustrazine.

## Materials and Methods

**Animals.** 24 male Wistar rats were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. The rats were 7 - 8 weeks of age at study initiation and weighing 200 - 240 g. The animals were housed in animal cages, 4 animals per cage in controlled temperature (19 - 23°C) and humidity (32 - 55%), with a 12 hour light/dark cycle. Filtered water and special diet formulation were provided *ad libitum*. The ligustrazine solution used in this study was prepared by adding deionized water in commercially available ligustrazine, up to a concentration of 163 mg/mL. The experiment was approved by the Ethics Committee of Hongqi Hospital of Mudanjiang Medical University. All animal experiments were carried out in accordance with the local legislation of animal experiments and laboratory standards of ethics to conduct appropriate research and with the EU Directive 2010/63/EU for animal experiments and Laboratories Code of Ethics.

### Reagents

Ligustrazine (Huanghua Pengfa Chemical Co., Ltd., China), paraformaldehyde (Shanghai Xinfan Biotech. Co., Ltd., China), alcohol (Shanghai Xinfan Biotech. Co., Ltd., China), hematoxylin eosin (Shanghai Xinfan Biotech. Co., Ltd., China).

The aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB), glutathione S-transferase (GST), malondialdehyde (MDA) kits were purchased

from Nanjing Jiancheng Biological Engineering Institute.

#### Experimental Methods

The rats required for the experiment were subjected to adaptive feeding for 1 week. The animals were randomly divided into the control group, DEN group - the experimental model for liver pre-cancerous state and DEN + ligustrazine group, 8 rats in each group.

Rats in the control group were injected with normal saline twice a week intraperitoneally (i.p.) for 15 weeks. The rats in the DEN group were injected with 55 mg/kg b.w. DEN i.p. twice a week for 15 weeks. The rats in the DEN + ligustrazine group received ligustrazine solution (163 mg/kg b.w.) daily by gavage and 55 mg/kg b.w. DEN i.p. twice a week for 15 weeks. During the treatment period, the activities, body posture, fur colour, feeding behaviour, of the rats in the three groups were observed daily.

After the end of 15 weeks, all rats were anesthetized with 3% pentobarbital i.p. The blood was collected from abdominal aorta. Serum was obtained by centrifugation and was used to evaluate the liver biomarkers. All rats were sacrificed by cervical dislocation and the liver was fixed using the neutral formalin for 24 h.

#### Biochemical analysis

The serum of the rats in each group was used to determine aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB), glutathione S-transferase (GST), malondialdehyde (MDA). These parameters were quantified using the Wright's colorimetry method, according to the instructions of the kit.

#### Histological analysis

Partial liver tissue was fixed in 4% paraformaldehyde solution for 24 hours, immersed in wax and embedded in paraffin. It was sliced in 4µm pieces, the sections were stained with haematoxylin eosin (HE) and subjected to histological observation using the Olympus Bx53 microscope (Olympus Corporation of Japan), detected by automatic liver function

analyser. The expression of the alpha-fetoprotein (AFP) in the control group, DEN model group and DEN + ligustrazine group was assessed by immune enzyme cell tissue chemical method (the reagent kit was purchased from Shanghai Bio Spectrum Technology Co., Ltd.) [19].

#### Statistical analysis

The data were analysed with Excel and SPSS 17.0 statistical software. Data were expressed as mean ± standard deviation. Comparisons between the three groups were performed using Student's t test, analysis of variance (ANOVA) and Wilcoxon test. The chi-square test was used in count, grade data.  $p < 0.05$  was established as the minimum significant difference.

## Results and Discussion

#### Rat liver function test results

The rats in the control group developed normally and all the liver biomarkers were normal. The rats in DEN group showed normal activities and lustrous fur for 1 to 5 weeks and their activities began to reduce starting from the 6th week, and showed rough fur and loss of appetite. The rats in DEN + ligustrazine group showed less activity, rough fur, less feeding.

The levels of AST, ALT, TB and GST in the DEN group were significantly higher than those in the control group ( $p < 0.01$ ). Compared with the DEN group, the levels of AST, ALT, TB, GST were significantly lower in DEN + ligustrazine group ( $p < 0.05$ ). Compared with the control group, the levels of MDA in the DEN group increased significantly ( $p < 0.01$ ). But the level of MDA in the DEN + ligustrazine group decreased significantly compared with the DEN model group ( $p < 0.01$ ). The recorded results are shown in Table I. The results showed that ligustrazine has an important role in liver protection, enzyme activity decrease and also the lipid oxidation was diminished in DEN + ligustrazine group.

**Table I**

Comparison of AST, ALT, TB, GST, MDA amounts in the three groups studied

Groups	AST (U/L)	ALT (U/L)	TB (µmol/L)	GST (U/L)	MDA (nmol/mL)
Control group	52.37 ± 4.04	61.01 ± 8.92	5.54 ± 0.49	628.83 ± 102.74	3.39 ± 1.45
DEN group	96.19 ± 6.87**	162.76 ± 8.20**	10.25 ± 1.51**	1301.35 ± 169.92**	8.27 ± 4.02**
DEN + ligustrazine group	68.72 ± 10.49*#	122.43 ± 11.54*#	7.38 ± 1.09*#	943.28 ± 72.67*#	4.32 ± 1.91*##

Note: \*\*  $p < 0.01$  vs. control group; #  $p < 0.05$  vs. DEN group; ##  $p < 0.01$  vs. DEN group.

#### Light microscopy in rat liver

The liver samples of the control group showed normal tissue structure and normal cell morphology for the liver lobule and the portal area (Figure 1). Liver lobules presented regular liver cell clusters. The adjacent liver plates were anastomosing and connected into a network, extending to the border around the liver lobule. Hepatocytes were polyhedron-shaped

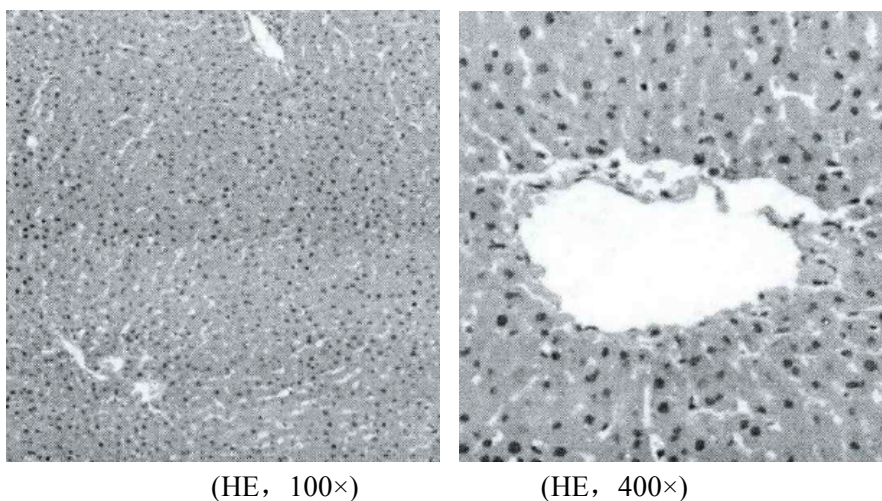
and the nucleus of the hepatocytes was round and located in the centre of the cell. The chromatin was sparse, the coloration was shallow, and the nuclear membrane was clear. Small droplets of fatty lesions were found. There were present endothelium and Kupffer cells in the hepatic sinusoids. The portal area included the hepatic artery, portal vein, bile duct and a small amount of connective tissue. There

were no significant degeneration and necrosis processes observed, no water spleen, bleeding, infiltration and bile stagnation, and no obvious lesions.

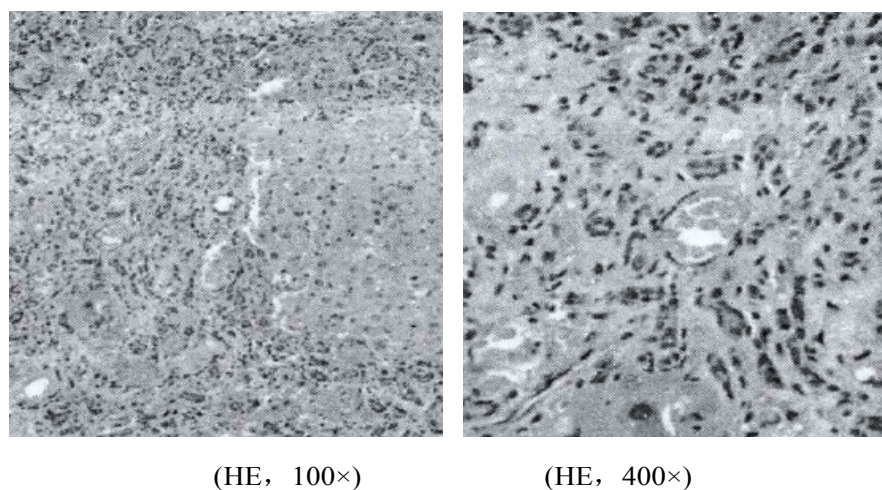
Compared with the control group (comparing Figure 1 and Figure 2), the hepatocytes metamorphism and nodular hyperplasia lesions of rats in the DEN group were obvious. In the same range of cells, the number of hepatocellular carcinoma (HCC) cells increased, and it was easy to find the typical hepatocytes. The main metamorphism regarded the

lipid droplets, cell oedema and necrosis, partial necrosis involving boundary of hepatocytes. The liver cells hyperplasia was visible in alkaline dyeing, the nuclear staining deepened, the volume was enlarged and the dual nuclei were common. The nuclear appearance obviously distorted.

Fibroplasia, inflammatory cell infiltration and bile duct hyperplasia were significant. Bile duct epithelial hyperplasia was characterized by atypia.



**Figure 1.**  
Hepatocytes of rats in the control group (HE- haematoxylin eosin staining)

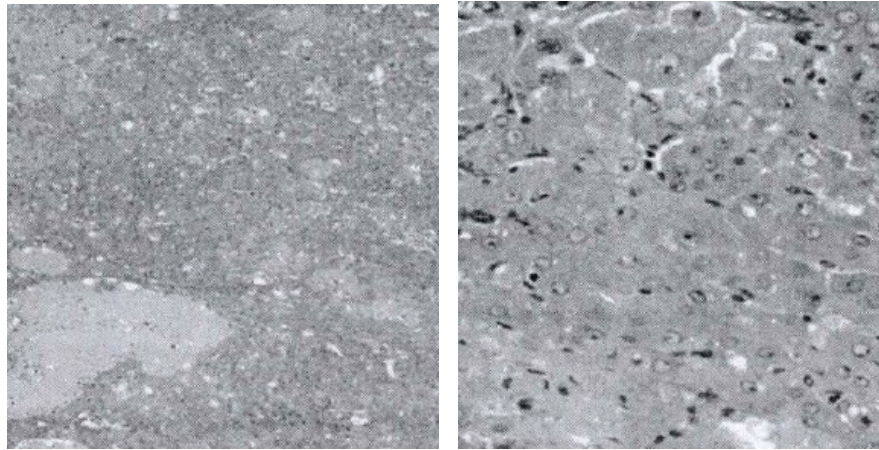


**Figure 2.**  
Hepatocytes of rats in DEN group

Compared with the DEN group (comparing Figure 2 and Figure 3), the pathological changes of the rats stem cells in the DEN + ligustrazine group were similar to those in the DEN group, but the degree was reduced. In the cell tissues with the same range, the number of HCC cells in DEN + ligustrazine group was less than that in the DEN model group. The dysplasia of the proliferating

cells decreased along with the number of the bi-nucleated cells. Dissolution necrosis rarely involved the lamellar lamina. The portal area had bile duct hyperplasia and lymphocytes infiltration. Hepatocytes showed decreased necrosis and the original liver cells in necrosis disappeared, being replaced by infiltrative lymphocytes or plasma cells leading to few cancer cells.





(HE, 100×)

(HE, 400×)

**Figure 3.**

Hepatocytes of rats in DEN + ligustrazine group

*Immunohistochemical assays*

The protein expression of the liver tissue of the control group, the DEN group, and the DEN + ligustrazine group was assessed by immunohistochemistry, as shown in Figure 4. The positive reaction of AFP protein was located in the cytoplasm of hepatocytes. It was weakly positive in the liver of the control group animals. However, the DEN group

and the DEN + ligustrazine group showed high AFT positive expression. The relative amount of liver protein positive expression in the samples of the three groups is shown in Table II. The results showed that AFP in the DEN group was significantly higher compared with the control group ( $p < 0.01$ ). The AFP of rats in the DEN + ligustrazine group was lower than in the DEN group ( $p < 0.01$ ).



**Figure 4.**

Immunohistochemical staining of AFP of rats in the three groups  
(HC, 40×, from left to right the control group, DEN group, DEN + ligustrazine group)

**Table II**  
Comparison of the relative expression of AFP of rats in the three studied groups

Groups	AFP level (IOD <sup>a</sup> )
Control group	40.21 ± 2.78
DEN group	97.62 ± 14.91 **
DEN + Ligustrazine group	71.36 ± 9.84 **##

Note: \*\*  $p < 0.01$  vs. the contro group; ##  $p < 0.01$  vs. the DEN group; <sup>a</sup> IOD - integrated optical density

Early detection of liver cancer predisposing factors such as family genetic disease, cancer risk signals, precancerous lesions, tumour self-examination, exploration of the mechanism of tumour development and early intervention that prevents the occurrence of cancer became an important part of liver cancer prevention

research [1]. Although the cause of disease prevention has achieved significant results, if the characteristics of precancerous lesions of the hepatocellular cannot be clearly defined, the targeted prevention and treatment will not be given accurately and effectively, which will bring great inconvenience and obstacles to the clinical prevention and treatment of liver cancer. Therefore, the understanding of the development of liver cancer and the studies on animal models that enlight the development processes of human liver diseases from inflammation to cancer have become an important prerequisite for research. ATL plays an important role in gluconeogenesis and aminoacid metabolism, and is an extremely sensitive indicator for the enhancement of liver cell membrane permeability. It is widely used as a

marker of liver damage [25]. AST mainly exists in the mitochondria of the cells, and the cytoplasmic expression is low. When the liver cells are seriously damaged, its activity in plasma will increase [22]. In our experiment, the levels of ATL and AST in the DEN + ligustrazine group were significantly inferior from those in the DEN group, which indicated that ligustrazine has certain inhibiting effect on hepatocytes injury. GST, TB are also sensitive indicators of liver injury [14, 24]. In this study, the activity of GST, TB in the DEN model group were significantly higher than the control group, while their levels in DEN + ligustrazine rats significantly reduced, indicating that ligustrazine has significant inhibition effects on hepatocyte injury. MDA is one of the final products of lipid peroxidation, reflecting the levels of free radicals. The MDA levels show the degree of the oxidative tissue damage [3]. The damage induced by free radicals or reactive oxygen species on cells is also one of the incentives of liver fat. So, anti-oxidant protection also has a certain role in the prevention of liver cancer. The MDA expression in rats from the DEN group was higher than the control group, and in DEN + ligustrazine group was lower than in DEN group, indicating that the mechanism of liver cancer should have an oxidative component, and the ligustrazine antioxidant activity is important. It can inhibit liver cell injury to a certain extent. AFP is synthesized in the human yolk sac and in liver. Studies have shown that AFP is mainly produced by the liver cancer cells themselves in liver cells carcinoma [12]. The results of the immunohistochemistry study showed that the content of AFP in rats from the DEN group was higher than in the control group, and the content of AFP in DEN + ligustrazine group was lower than in the DEN model group. We can conclude that ligustrazine has a certain effect on the prevention of early liver cancer.

## Conclusions

This study showed that ligustrazine reduces the DEN-induced liver injury degree in rats with hepatic precancerous lesions, and has a role in protecting the liver. Ligustrazine has a positive effect on inhibiting the development of hepatic precancerous lesions in rats.

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