



# The Protective Effects of Hepatopoietin Cn (HPPCn) on Acute Liver Injury

Na Li<sup>1#</sup>, Feng-Jiao Liu<sup>1#</sup>, Chun-Xia Sun<sup>2</sup>, Dan-Dan Li<sup>2</sup>, Jian Li<sup>1</sup>, Mei-Hua Qu<sup>1\*</sup>, Chun-Ping Cui<sup>3\*</sup> and Dajin Zhang<sup>2\*</sup>

<sup>1</sup>Department of Pharmacy, Weifang Medical University, China

<sup>2</sup>Department of Medical Sciences, Sixth Medical Center of PLA 301 Hospital, China

<sup>3</sup>Department of Protein Sciences, National Center of Protein Sciences (Beijing), China

\*Both Authors contributed equally

## Abstract

**Objective:** To observe the protective role of HPPC non acute liver injured.

**Methods:** Six hours after 10 mmol/L CCl<sub>4</sub>, 150 mmol/L ethanol or 0.6 mmol/L H<sub>2</sub>O<sub>2</sub> treatment, human hepatoma SMMC7721 cells were incubated with 10, 100, 200 ng/ml recombinant human HPPCn protein (rhHPPCn) for another 24 hrs. The cell survival rate was analyzed by CCK-8 assay. The CCl<sub>4</sub> induced apoptosis of SMMC7721 cells was detected by flow cytometry. Then the levels of GOT, GPT, MDA, LDH, GSH-PX and SOD in SMMC7721 cells lysates or cell culture supernatant were detected. SMMC7721 cells were treated with different concentrations of rhHPPCn (0, 10, 100 ng/ml). The expression of Cell proliferation indexes (BrdU and PCNA) were detected by Immuno Histo Chemistry (IHC). Acute liver injury mouse model was established by a one-time tail vein injection of 20% CCl<sub>4</sub> at a volume of 5 ml/kg body weight. At one hour after the injection, 2.5 mg rhHPPCn/12h/kg body weights were administered through tail vein injection. The serum levels of GOT and GPT were detected and pathological changes in liver were evaluated. The expression changes of PCNA were observed by IHC.

**Results:** rhHPPCn could increase the survival rate of SMMC7721 cells and inhibit chemical toxics-induced cell apoptosis. The cell supernatants levels of GOT, GPT, MDA, LDH were significantly reduced, while GSH-PX and SOD levels significantly increased after rhHPPCs treatment in the CCl<sub>4</sub> treated SMMC7721 cells. The expression of BrdU and PCNA was increased by concentration dependent, and this effect, which indicates that rhHPPCn, can promote cell proliferation. In addition, rhHPPCn has a liver protective effect against liver injuries *in vivo*. It significantly reduced serum GOT, GPT levels in CCl<sub>4</sub> induced acute liver injury mouse in a time and concentration-dependent manner, and increased the expression of PCNA in liver significantly.

**Conclusion:** rhHPPCn protects the hepatocytes from chemical agents by promoting proliferation and inhibiting apoptosis both *in vivo* and *in vitro*. Our study can provide new insights for clinical treatment of acute liver injury.

## Introduction

HPPCn is a growth factor isolated from the Hepatic Stimulator Substance (HSS) of newborn calf, which promotes hepatocytes proliferation. Sequence analysis showed that HPPCn belongs to ANP32 (Acidic/leucine-rich nuclear phosphor protein 32) family [1,2]. ANP32 family members are involved in various biological processes such as cell proliferation, differentiation and apoptosis, and are related to tumorigenesis and drug resistance [3]. HPPCn is the only known member of the ANP32 family that can perform the role of cytokines outside the cell [4]. It was reported that recombinant human HPPCn protein (rhHPPCn) could stimulate hepatocytes proliferation *in vitro* and *in vivo*, by activating signaling pathways that includes sphingosine kinase-1 and extracellular signal regulated kinase [5,6]. Acute liver injury is a common disease caused by various reasons, 40% were drug-induced liver dysfunction, which requires a complex liver regeneration process for recovery [7]. The liver regeneration process involves many liver stimulating factors, growth factors, cytokines and their downstream signaling [8]. Most of the factors found target a variety of cells and organs, lacking specificity for liver regeneration process. However, HPPCn is a well-defined hepatocytes growth factor with specific hepatic stimulating activities in partially hepatectomized mice [4,5,9]. Mice

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### \*Correspondence:

Da-Jin Zhang, Department of Medical Sciences, Sixth Medical Center of PLA 301 Hospital, Beijing 100048, PR. China,

E-mail: dajinzhang@sina.com

Mei-Hua Qu, Department of

Pharmacy, Key Laboratory of Applied

Pharmacology, Weifang Medical

University, Wei Fang, Shandong

261053, PR. China,

E-mail: qumeihua2016@163.com

Chun-Ping Cui, Department of Protein

Sciences, State Key Laboratory of

Proteomics, National Center of Protein

Sciences (Beijing), Beijing Institute of

Lifeomics, 100850 Beijing, China,

E-mail: cui\_chunping2000@aliyun.com

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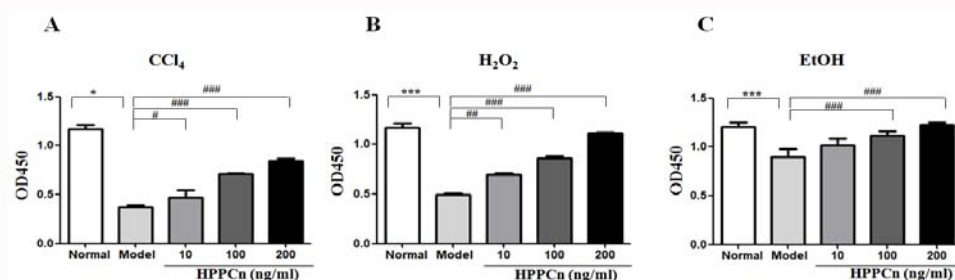
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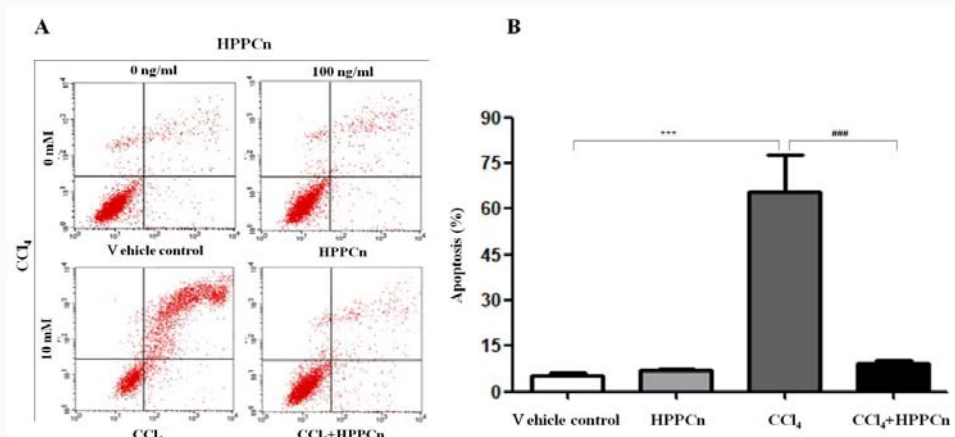
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**Figure 1:** rhHPPCn increased the survival of SMMC7721 cells injured by toxic substances. **(A)** Injury of SMMC7721 cells were induced by 10 mmol/l CCl<sub>4</sub>, **(B)** 0.6 mmol/L H<sub>2</sub>O<sub>2</sub>, **(C)** 50 mmol/l alcohol for 6 hrs prior to treatment with indicated concentrations of rhHPPCn. It was showed that rhHPPCn could increase the survival of SMMC7721 cells in a dose-dependent manner. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001: vs. Control. #p<0.05, ##p<0.01, ###p<0.001: vs. Model.



**Figure 2:** rhHPPCn inhibits apoptosis of SMMC7721 cells damaged by CCl<sub>4</sub>. **(A)** The cells apoptosis image of Vehicle control group, HPPCn group (100 ng/ml HPPCn), CCl<sub>4</sub> group (10 mmol/l CCl<sub>4</sub>) and CCl<sub>4</sub>+HPPCn group (10mmol/l CCl<sub>4</sub>, 100ng/ml HPPCn) were detected by FITC-conjugated Annexin V and PI by flow cytometry. **(B)** Statistical chart of apoptosis rate in four groups (Vehicle control group, HPPCn group, CCl<sub>4</sub> group and CCl<sub>4</sub>+HPPCn group). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001: vs. Vehicle control group. #p<0.05, ##p<0.01, ###p<0.001: vs. CCl<sub>4</sub> group.

experiments *in vivo* showed that rhHPPCn had no significant effect on various tissues and organs in normal mice, including liver. However, HPPCn expression in liver was significantly increased in partially hepatectomized mice [9,10]. In addition, rhHPPCn can stimulate DNA replication and Erk phosphorylation processes in PH mice hepatocytes. Our previous studies have shown that rhHPPCn and its family members can significantly reduce the damage of aflatoxin and alcohol on hepatocytes and promote hepatocytes proliferation [11]. In addition, rhHPPCn can delay alcohol or CCL4-induced chronic liver fibrosis [2]. Therefore, we hypothesized that rhHPPCn may play an important role in the protection against acute liver injury. This study used CCL4, ethanol and H<sub>2</sub>O<sub>2</sub> to induce damage hepatic cell model and CCl<sub>4</sub> alone to induce acute liver injury mice models, and observed the protective effects of rhHPPCn on acute liver injury to discover its underlying mechanisms.

## Materials and Methods

### Chemicals

The rhHPPCn protein was obtained by purification with Nickel Ion Column Affinity Chromatography from high cell density cultivation of the *Escherichia coli* strain BL21 (DE3)/pCold II-HPPCn as previously described. Mouse anti-rhHPPCn monoclonal antibody was prepared by the Beijing C&N International Sci-techCo (Beijing, China). Toxic substances including Ethanol (C<sub>2</sub>H<sub>5</sub>OH), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and carbon tetrachloride (CCl<sub>4</sub>) were purchased from SionphrmCo. (Beijing, China).

### Cell model

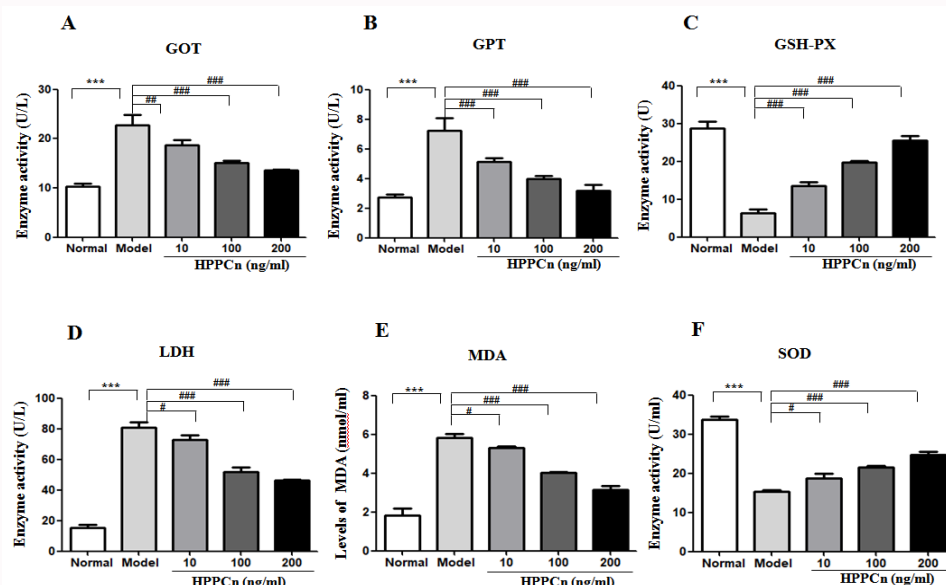
Human hepatoma cells SMMC7721 were obtained from Chinese Academy of Sciences Cell Bank (Shanghai, China), and cultured in RPMI1640 (Gibco, Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, UT, USA), 100 IU/mL penicillin and 100 mg/mL streptomycin (Hyclone, UT, USA). Cells were maintained in a humidity incubator at 37°C (Thermo Fisher Scientific, MA, and USA) with 5% CO<sub>2</sub>.

### Animal model of acute liver injury with CCl<sub>4</sub>

To study the effect of rhHPPCn in the acute liver injury, 20 g to 22 g male Balb/c mice were randomly separated into 7 groups (8 in each group). A single tail vein injection of 20% CCl<sub>4</sub> with a volume of 5 ml/kg body weight was used to establish acute liver injury in the mice model; untreated mice were used as normal control.

### Cell viability by CCK-8 Assay after toxic damage and rhHPPCn protein stimulation

SMMC7721 cells were digested by 0.05% trypsin (containing 0.02% EDTA), 5×10<sup>4</sup> cells μL<sup>-1</sup> were resuspended in 100 μL RPMI1640 were cultured in 96-well culture plate per well at 37°C for 24 hr in cell culture incubator. Then, final concentration of 150 mmol/L ethanol, 0.6 mmol/L H<sub>2</sub>O<sub>2</sub> or 10 mmol/L of CCl<sub>4</sub> (1% DMSO preparation) were added respectively and incubated for another 6 hrs. Different concentrations rhHPPCn (final concentration of 10 ng/mL, 100 ng/mL, and 200 ng/mL) were added to the treated cells and cultured for another 24 hr. 10 μL cell counting kit-8 solution (CCK-8,



**Figure 3:** rhHPPCn reduced  $\text{CCl}_4$  induced-cytotoxicity in SMMC7721 cells. (A,B,D,E) After treated with different concentrations of rhHPPCn for 24 hrs, the cell culture supernatants were collected, and the cells were harvest by lysates. It was showed that  $\text{CCl}_4$  induced descending GOT, GPT, LDH and MDA and elevating GSH-PX and (C,F) SOD. rhHPPCn treatment significantly reduced the changes of these cytotoxicity indexes in a dose-dependent manner. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ : vs. Control. # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$ : vs. Model.

Dojindo Biotechnology, Kumamoto, Japan) was added to each well, and the plates were incubated for 1 h at  $37^\circ\text{C}$  in humidity incubator. The absorbance at 450 nm was measured by Micro-plate Reader (Bio-Rad, Hercules, CA, USA).

### Apoptosis detection

The cells were treated in the same manner as above, and then inoculated into 6 well plates with  $3.6 \times 10^5$  cells per well. Cells were divided into four groups: Vehicle control group, rhHPPCn group (100 ng/ml rhHPPCn),  $\text{CCl}_4$  group (10 mmol/l  $\text{CCl}_4$ ) and  $\text{CCl}_4$ +rhHPPCn group (10 mmol/l  $\text{CCl}_4$ , 100 ng/ml rhHPPCn). The cells were cultured overnight. The  $\text{CCl}_4$ +rhHPPCn group and  $\text{CCl}_4$  group were treated with 10 mmol/l  $\text{CCl}_4$  for 6 hrs; then  $\text{CCl}_4$ +rhHPPCn group was stimulated with 100 ng/ml rhHPPCn for 24 hrs. Flow cytometry with Annexin V was used for the detection of cell apoptosis. Apoptotic cells were detected using FITC-conjugated Annexin V and Propidium Iodide (PI). The cells were washed twice with cold PBS and resuspended in an Annexin V-binding buffer containing 10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 140 mmol/l NaCl, and 5 mmol/l  $\text{CaCl}_2$  at a concentration of  $1 \times 10^6$  cells/mL. The suspension (100  $\mu\text{l}$  containing  $1 \times 10^6$  cells), 5  $\mu\text{l}$  of Annexin V-FITC and 10  $\mu\text{l}$  of PI were added into a 5 ml culture tube. The tube was gently vibrated and incubated for 15 min in the dark at room temperature. After a binding buffer (400  $\mu\text{l}$ ) was added into the tube, the cells were analyzed by flow cytometry.

### Detection of cytotoxicity relevant indicators in SMMC 7721 cells

The experimental method of  $\text{CCl}_4$  induced cell injury was performed in the same manner as before. After 6 hrs of  $\text{CCl}_4$  treatment, rhHPPCn with final concentration of 10 ng/ml, 100 ng/ml and 200 ng/ml were added respectively to the cells. Three duplicate wells were set in the experiment. 24 hrs later, Glutamic Oxaloacetic Transaminase (GOT), Glutamic-Pyruvic Transaminase (GPT) and Super Oxide Dismutase (SOD) levels were detected after cell disruption using commercially available kits (Njicbio, Nanjing,

China). Glutathione peroxidase (GSH-PX), Lactate dehydrogenase (LDH) and Malondialdehyde (MDA) levels in cell supernatant was measured assay kits (Njicbio, Nanjing, China).

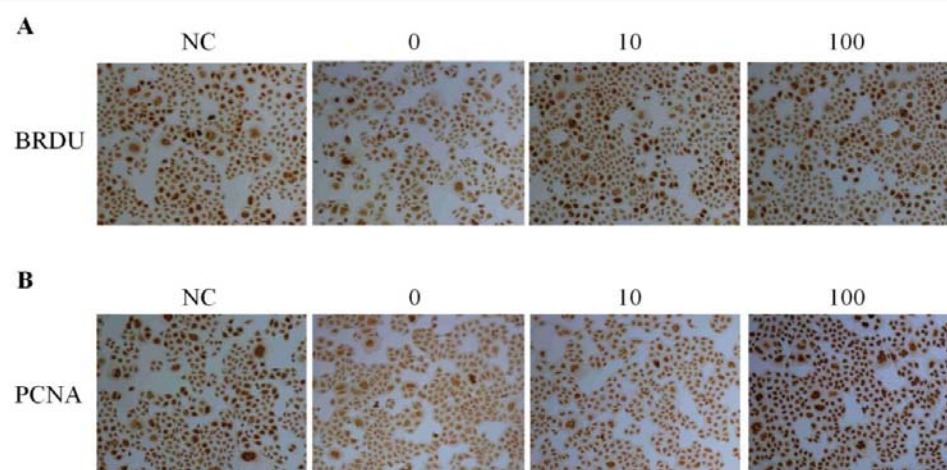
### Observation of serum GOT and GPT levels of the mice

To invest whether rhHPPCn protection of acute liver injury is dose-dependent. Gradient concentration rhHPPCn at (1,5,15,25,50  $\mu\text{g}$  rhHPPCn/12h/20g body weight) was injected by tail vein respectively 1 hr after  $\text{CCl}_4$  injection. PBS injection used as model control. The animals were sacrificed 24 hrs after rhHPPCn injection. Blood were collected through medial canthus vein of each mice. The serum GPT and GOT levels were measured by ELISA kits (Njicbio, Nanjing, China). To invest the time course of rhHPPCn effect on the acute liver injury, 50  $\mu\text{g}$  of rhHPPCn/12h/20g body weight was injected via tail vein one hour after the setting model. Blood taken from medial canthus vein of animals at 0,12,24,36, and 48 hrs after the treatment. Serum level of GPT and GOT were detected by ELISA kits.

### HE staining and immunohistochemistry assay

SMMC7721 cells in logarithmic growth phase were inoculated in the 96-well culture plate according to the concentration of  $5 \times 10^3$ /well. After the cells were adhered to the wall, rhHPPCn with a final concentration of 0,10,100 ng/ml was added in well for 48 hr. Before collecting samples from 2 hr, the cells were treated with 10  $\mu\text{mol/L}$  BrdU and were fixed 4% para-formaldehyde, 0.5% TritonX-100 perforated for 20 min on ice, Endogenous peroxidase was removed by 3%  $\text{H}_2\text{O}_2$  for 10 mins, and 10% FBS was blocked for 2 hrs. Antibodies against BrdU and PCNA were added respectively, and overnight at  $4^\circ\text{C}$ . Secondary antibodies labeled with HRP were added the next day. DAB was stained and observed under microscope (IX71, Olympus, Japan). The liver specimens were dewaxed in xylene; rehydrated in ethanol, and washed with PBST for 10 mins, and Hematoxylin and Eosin (HE) staining was performed. IHC staining was performed using tissue sections. Liver specimens were treated with 3% hydrogen peroxide for 10 mins, blocked with 10% FBS for 2 hrs, then tissue slices were incubated with anti-PCNA antibodies (1:100, ab29,





**Figure 4:** Effects of rhHPPCn recombinant protein on the proliferation of SMMC7721 cells (100x). **(A)** IHC results showed that the expression of Brdu increased with the concentration of rhHPPCn increasing. **(B)** The expression of PCNA was increased. HPPCn could promote the proliferation of SMMC7721 cells.

Abcam, Cambridge, MA). Tissue slices were applied HRP-conjugated secondary antibody for 1h at room temperature. Non immune rabbit serum was used for negative controls. After rinse with PBST three times for 2 mins, slices were incubated with Di Amino Benzidine (DAB) for 5 mins at room temperature. The location and level of protein expression were observed and analyzed under microscope (IX71, Olympus, Japan).

### Statistical analysis

All results are expressed as means  $\pm$  SD. Multiple group comparisons were performed by Student's t-test;  $P < 0.05$  was considered statistically significant.

## Results

### rhHPPCn increased the survival of SMMC7721 cells injured by toxic substances

The SMMC7721 cell viability after 6 hrs treatment of  $\text{CCl}_4$ , EtOH or  $\text{H}_2\text{O}_2$  was detected by CCK-8 assay. As showed in Figure 1A, the cell viability significantly reduced after 6 hrs treatment by  $\text{CCl}_4$ . After another 12 hrs incubating with rhHPPCn at a concentration of 10,100,200 ng/ml, the surviving rate of cell in rhHPPCn-treated group increased 1.25, 1.89 and 2.26 times, respectively, comparing to the  $\text{CCl}_4$ -treated model cells. The number of rhHPPCn-treated cells increased significantly, and this effect was more pronounced with increasing rhHPPCn concentration in a dose-dependent manner (Figure 1A). The protection effect of rhHPPCn from EtOH and  $\text{H}_2\text{O}_2$  in SMMC7721 cells displayed similar pattern of  $\text{CCl}_4$ -treated SMMC7721 cells (Figure 1B,1C).

### rhHPPCn inhibits apoptosis of SMMC7721 cells

It has been demonstrated that  $\text{CCl}_4$  exposure is linked to hepatocytes apoptosis, and rhHPPCn can reverse the apoptosis caused by this chemical injury (Figure 2A).  $\text{CCl}_4$  induced apoptosis was significantly inhibited in cells treated with 100 ng/ml rhHPPCn (Figure 2A). The cell apoptosis rate in the rhHPPCn group was not significantly different compared with Vehicle control group ( $6.95 \pm 0.67\%$  vs.  $5.39 \pm 0.86\%$ ), this indicates that rhHPPCn has no cytotoxicity. In the  $\text{CCl}_4$  group,  $65.59 \pm 12.30\%$  of SMMC7721 cells was apoptotic (Figure 2B). While the apoptosis rate of  $\text{CCl}_4$ +rhHPPCn group was decreased to  $9.18 \pm 1.04\%$ .

### HPPCn protected SMMC7721 cells from chemical toxicants

It was showed that 10 mm/l  $\text{CCl}_4$  could induce cytotoxicity in SMMC7721 cells. The levels of GOT, GPT, LDH and MDA elevated apparently. And the levels of GSH-PX and SOD descended obviously. When treated with rhHPPCn, the levels of these damage indicators LDH, MDA, GOT and GPT were decreased, and the levels of these protective indicators GSH-PX and SOD in  $\text{CCl}_4$ -damaged SMMC7721 cells were increased in a dose-dependent manner (Figure 3).

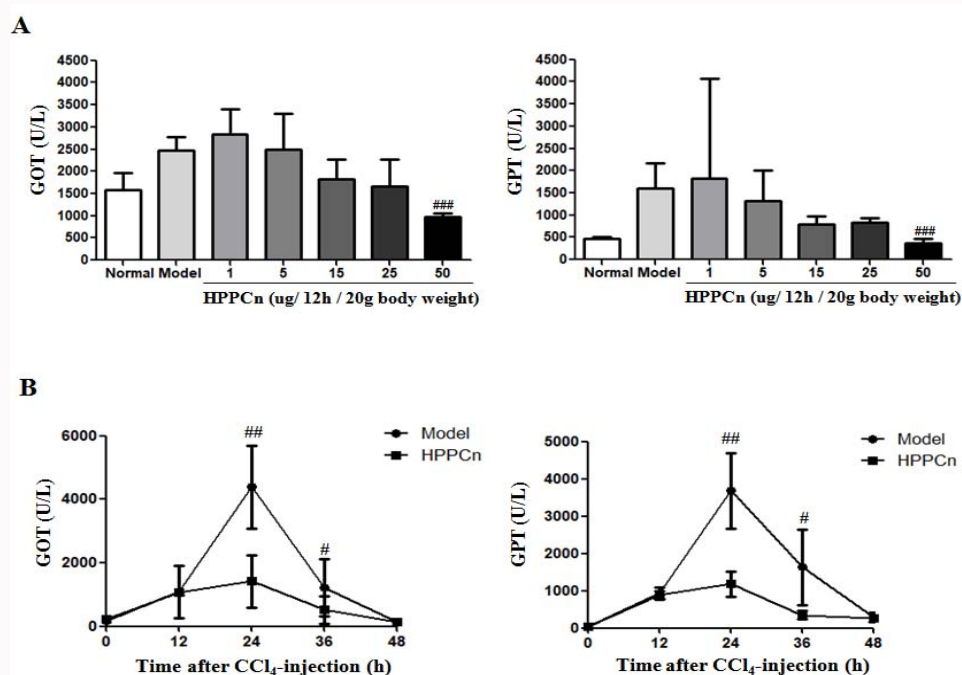
### HPPCn promotes SMMC7721 cells proliferation

HPPCn could significantly promote cell proliferation. The results of IHC showed that the expression of Brdu in SMMC7721 cells which were treated with rhHPPCn were increased, this was a concentration-dependent effect. This result suggested that rhHPPCn could promote the synthesis of DNA in SMMC7721 cells. Meanwhile, the expression of PCNA in SMMC7721 cells also increased with the increase of rhHPPCn concentration, suggesting that rhHPPCn could also promote the proliferation of SMMC7721 cells.

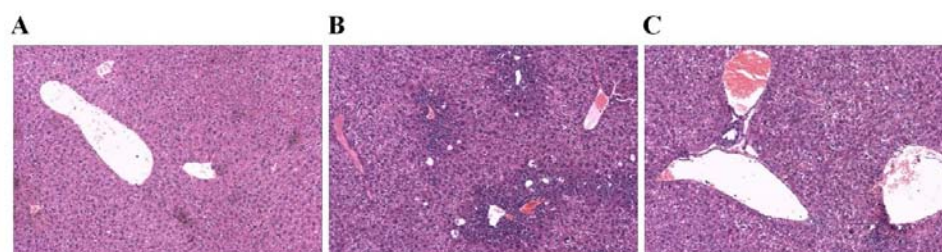
### HPPCn protected $\text{CCl}_4$ -induced acute liver injury mice in a concentration and time course-dependent manner

Mice with acute liver injury induced by  $\text{CCl}_4$  were treated with different concentrations of rhHPPCn. After 24 hrs, serum GPT and GOT levels were detected. The results showed that rhHPPCn could reduce serum GPT and GOT levels in mice with  $\text{CCl}_4$ -induced acute liver injury (Figure 4). When the dose reached 50  $\mu\text{g/kg}$ , there was a significant difference compared with the Model group. Therefore, the relationship between the protective effects of 50  $\mu\text{g/kg}$  rhHPPCn on acute liver injury induced by  $\text{CCl}_4$  and time-dependent were explored. The results showed that the levels of GPT and GOT in mice increased rapidly after  $\text{CCl}_4$  treatment, peaked at 24 hr and remained at high level even after 36 hr, however, it returned to normal level after 48 hr. This indicated that it was the peak of injury within 48 hrs after acute injury, and the protective effect on this stage was most important. The serum GPT and GOT levels of mice were significantly decreased by more than 3 times at 24 and 36 hrs after  $\text{CCl}_4$ -induced acute liver injury. The results showed that rhHPPCn had protective effect on  $\text{CCl}_4$ -induced acute liver injury in mice. After mice with  $\text{CCl}_4$ -induced acute liver injury were treated with rhHPPCn, the serum

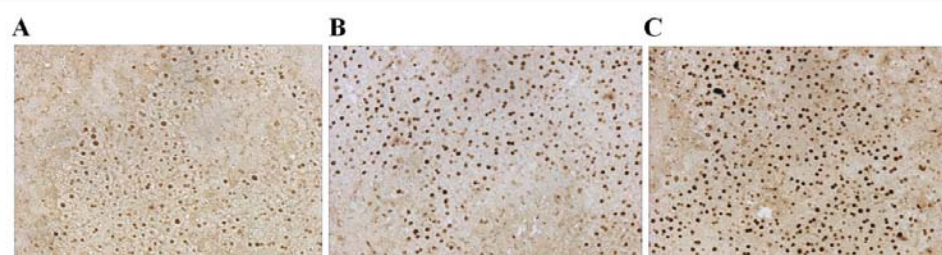




**Figure 5:** The effects of rhHPPCn none the serum levels of GOT and GPT in CCl<sub>4</sub>-induced acute liver injury mice. The serum levels of GOT and GPT in CCl<sub>4</sub> induced acute liver injury mice were elevated dramatically comparing with the mice in normal group. It was showed that rhHPPCn could decrease the levels of GOT and GPT in serum of mice in a dose- (A) and time- (B) dependent manner. After acute liver injury, treatment of 2.5 mg/12h/kg body weight rhHPPCn had the most significant effects at 24 and 36 hours compared with the control group. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001: vs. Control. #p<0.05, ##p<0.01, ###p<0.001: vs. Model.



**Figure 6:** rhHPPCn reduced the injury of liver tissue in CCl<sub>4</sub>-induced acute liver injury mice. (A) HE staining of the liver tissue in control group, (B) HE staining of the liver of CCl<sub>4</sub> induced acute liver injury mice, (C) HE staining of the liver of CCl<sub>4</sub> induced acute liver injury mice and treated with rhHPPCn at 2.5 mg/12h/kg body weight for 24 hrs.



**Figure 7:** rhHPPCn promoted the proliferation of liver cells in CCl<sub>4</sub>-induced acute liver injury mice. (A) The expressions levels of PCNA detected by immuno histochemistry in the liver tissues of the mice in the model group, (B) Acute liver injury model treated with rhHPPCn at 1.25 mg/12h/kg body weight, (C) Acute liver injury model treated with rhHPPCn at 2.5 mg/12h/kg body weight.

GPT and GOT levels were significantly decreased at 24 and 36 hrs. This indicated that rhHPPCn has a protective effect on CCl<sub>4</sub>-induced acute liver injury in mice.

#### HPPCn reduces CCl<sub>4</sub>-induced liver injury in mice

The HE staining showed in Figure 5A, 24 hrs of CCl<sub>4</sub> treatment induced mice liver cells injury by edema, vacuolization, a little necrosis

around small blood vessels, and inflammatory cells infiltrated (Figure 5B). rhHPPCn incubation at 2.5 mg/12h/kg incubation significantly reduced the injury of liver cells, and only some hepatocytes edema and a small amount of inflammatory cells exuded (Figure 5C). That indicated the rhHPPCn could obviously reduce the liver-injured by CCl<sub>4</sub>.

## HPPCn promotes CCl<sub>4</sub>-induced hepatocytes proliferation in acute liver injury mice

Different doses of rhHPPCn (0, 1.25, 2.5 mg/12h/kg body weight) were administrated at 1 hour after CCl<sub>4</sub>-induced acute liver injury. IHC analysis showed that rhHPPCn could significantly promote the expression of PCNA in injured hepatocytes ( $p < 0.05$  vs. vehicle). Compared with CCl<sub>4</sub>-treated group without rhHPPCn treatment (Figure 6), pretreatment with rhHPPCn protein significantly prevented the accumulation of liver injury in human hepatocytes induced by 10 mM/LCCL4 in a dose-dependent manner.

## Discussion

The liver is an important organ of the body for it takes part in a variety of metabolic functions, such as de-oxidation, storage of glycogen, synthesis of secreted proteins. Liver injuries (induced by toxins, viruses, surgery etc.) subject the surviving hepatocytes to its physiological functions and restore the liver volume by liver regeneration. Hepatic regulatory factors including rhHPPCn play an important role in the process of liver regeneration [12-14]. The survival of a patient with more than 80% of hepatocytes damage depends entirely on liver regeneration. However, liver regeneration is often insufficient in severe liver disease. High mortality rate was often found in fulminant hepatitis patients with hepatocytes volume of less than 12%. Therefore, the search for specific and safe liver stimulating factors is of great significance for the treatment of acute liver injuries. Many cytokines involved in liver regeneration also demonstrate protective effects against different liver damages. HGF is an effective DNA synthesis stimulator in hepatocytes, which also protects the liver against fibrosis and other types of damage [9,15,16]. HSS protects the liver from the hepatic agent CCl<sub>4</sub> and galactosamine against fibrosis [17,18]. Liver regeneration enhancer ALR can inhibit liver atrophy induced by Eck's fistula (investigation of portal vein inferior vena cava anastomosis performed by liver metabolism) [19-22]. The results of the study showed that these factors can effectively protect the liver from further damages *in vivo* and improve animal survival by promoting hepatocytes DNA synthesis and liver regeneration. However, these hepatic regenerative cytokines are limited in their application due to lack of specificity or unclear composition and mechanism. LaBrecque et al. [23] reported that HSS is only present in the liver of newborn mammals or regenerative liver, which not only stimulates the synthesis of liver cells and mitosis *in vitro*, but also promotes liver regeneration in some liver resected animals. It has no such effects on other organs, such as bone marrow, spleen, and kidney [1,24]. rhHPPCn was isolated and identified from the newborn calf liver HSS through the combination of traditional biochemical separation methods and newly developed proteomics research techniques [2]. Studies have shown that the expression level of rhHPPCn after partial hepatectomy in mice is temporally dynamic [10]. Similar to HSS, rhHPPCn's stimulating proliferative activity exhibits organ specificity. Not only does it stimulate hepatocytes cultured *in vitro* to initiate DNA synthesis and mitosis, it also promotes liver regeneration in some liver resected animals *in vivo*, and has no effects on other organs. CCl<sub>4</sub> is a pro-hepatic toxicant and is considered to be a classic chemical inducer of the liver injury model commonly used for liver-protecting drug screening. CCl<sub>4</sub> mainly destroys the liver cell membrane through the oxidative stress reaction caused by its free radical metabolites, and increases the permeability of the cell membrane, thereby causing liver damage. After CCl<sub>4</sub> enters the body, it is metabolized to trichloromethyl group under the action of liver microsomal enzyme. Trichloromethyl group can destroy

the structure of liver cell membrane and increase the permeability of hepatocytes membrane, resulting in the release of intracellular enzymes GPT and GOT from the cell and causes an increase in GPT and GOT levels in the blood. Therefore, GPT and GOT in serum can directly reflect the degree of liver damage. This study established a model of acute liver injury induced by CCl<sub>4</sub> in mice, and observed the protective effect of rhHPPCn on liver injury. The results showed that the serum GPT and GOT level increased significantly in the CCl<sub>4</sub>-induced acute liver injury model group, and the rhHPPCn administration group could significantly reduce the serum GPT and GOT levels of CCl<sub>4</sub>-induced acute liver injury mice in a concentration and time dependent manner. HE staining tissue sections showed that rhHPPCn can significantly reduce inflammation and necrosis of damaged liver tissue, which indicates the protective effect of rhHPPCn non-chemical toxic-induced liver injury. CCl<sub>4</sub> undergoes redox reactions in the body to generate a large number of free radicals, which attack the unsaturated fatty acids on the cell membrane and induce lipid peroxidation. Lactate De-Hydrogenase (LDH) is a cytoplasm enzyme, which is released when the cell membrane is damaged. The amount of LDH detected can be used as an indicator for determining the number of dead cells. MDA is one of the final products of lipid peroxidation, which is accumulated in the process of CCl<sub>4</sub>-induced liver injury. It then combined with bio macro molecules to form aldehyde, and further undermines the structure and function of liver cell membrane. SOD, an effective Metallo enzyme, can catalyze the disproportionate of superoxide anion to H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>. GSH-PX catalyzes the reduction of toxic peroxides into non-toxic hydroxy compounds, while also reducing H<sub>2</sub>O<sub>2</sub> and hydroperoxides to water, removing lipid hydrogen peroxide from the cell membrane, thereby terminating lipid peroxidation. The results of this study showed that the rhHPPCn treatment group can effectively reduce the levels of MDA, LDH, GPT and GOT in human hepatoma cells SMMC7721 after CCl<sub>4</sub> treatment, and increase the levels of SOD and GSH-PX (Figure 7). This study showed that rhHPPCn can attenuate the toxicity of CCl<sub>4</sub>, ethanol and H<sub>2</sub>O<sub>2</sub> chemical toxics on SMMC7721 cells. rhHPPCn significantly increased the expression of PCNA in the liver of mice and SMMC7721 cells with CCl<sub>4</sub> treatment, and significantly inhibited the apoptosis of damaged cells. These results indicate that rhHPPCn has a protective effect on acute liver injuries, which reduces toxic damage and protects the liver. Its mechanism may be related to the involvement of rhHPPCn in regulating liver regeneration and inhibiting apoptosis. Previous studies have shown that HPPCn can be secreted by non-canonical pathways. HPPCn may act as a cytokine in an autocrine or paracrine form to interact with receptors on the surface of hepatocytes, activating signaling pathways like SPK, Erk1/2 and Jak-Stat3 to promote hepatocytes proliferation and liver protection. For example, HPPCn significantly increases the phosphorylation of Erk1/2 in the liver of some hepatectomized mice [25]. It was reported that HPPCn attenuated oxidative injury and fibrosis induced by ethanol feeding and that the SphK1/S1P/S1PRs signaling pathway contributes to the protective effect of HPPCn on hepatocytes apoptosis and HSC activation [26]. HPPCn activates signaling pathways involved in the survival of HCC Cells and up-regulates myeloid cell leukemia-1 (Mcl-1) expression via MAPK, SPK1 pathways [27,28]. Although studies suggested that HPPCn may be associated with tumorigenesis, low HPPCn expression may inhibit HepG<sub>2</sub> proliferation and tumorigenesis *in vivo*, but there is no direct evidence that HPPCn protein is directly or *in vivo* to directly cause a high risk of tumors. Moreover, HPPCn protein, one of the main active ingredients obtained from HSS, has good stability and is easy

to prepare. In addition, a large amount of experimental data indicates that extracellular administration of rhHPPCn has little proliferative effect on normal mammalian cells without toxicant treatment including tumor cells [29]. Therefore, it is worth exploring the short-term application of HPPCn to improve the prognosis and reveal the underlying mechanism of its action.

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