Herbal medicine for liver protection in experimental animals - a histochemical, pathological study

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Pien Tze Huang (PTH) is a mixture of several agents of traditional medicine prescribed over 600 years ago by a retired Court Physician, for conditions of liver diseases in southern China. For all these hundreds of years till the present day, PTH traditional medicine has been widely taken orally by the ethnic communities not only in China but also in the Southeast Asia as a hepatoprotective drug. Previous animal studies on this traditional drug revealed that it could indeed protect liver damage against carbon tetrachloride (CCL₄) insults in mice model and hepatoma culture. After drug treatment of PTH, increased level of markers responsible to tackle superoxide formation and upregulated immune activities were evident [1]. The present study attempted to evaluate the further hepatoprotective effect of this drug against bile duct ligation and acute intoxication of alcohol in the mouse model, employing biochemistry and histochemistry, along with histopathological observations. After bile duct ligation and acute alcohol treatment, both lactic dehydrogenase (LDH) positive cells (indicating necrosis) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells (indicating apoptosis) went up in these experimental animals, along with an increase in transaminase. Histopathological observation indicated increase in fibrosis starting 10 days after bile duct ligation or 7 days after acute alcohol model. However, in those animals with PTH treatment which had either bile duct ligation or acute alcohol treatment, the number of necrotic and apoptotic cells decreased when compared with those experimental animals without PTH treatment. Concurrently, there was also a downregulation of the transaminase and the extent of fibrosis after PTH treatment in the experimental animals of both ligation and alcohol treated groups. Transforming growth factor beta (TGF-beta) positive cells also increased in number in the bile duct ligated mice but not in the acute alcohol treated mice. In the former group, PTH treatment was effective in the downregulation of the increase of TGF-beta cells in the liver. In addition, PTH treatment enhanced the proliferation of cells in the liver after bile duct ligation and acute alcohol treatment, thus helpful in regeneration as well. On the whole, the PTH treatment which had been used for more than 600 years appeared to have definitive protective effects on the liver after injury as demonstrated in this study of the mice model.

Keywords: traditional Chinese medicine; Pien Tze Huang; liver; hepatoprotective; histochemistry;

1. Introduction

Pien Tze Huang (PTH) is a traditional herbal medication used as early as 1555 in the Ming dynasty to treat liver ailment and to this day is still used widely in Asia for liver diseases and hepatoprotection. The composite had been tested in rodent models using carbon tetrachloride (CCL₄) to produce injury on the liver. Subsequent analysis demonstrated some forms of hepatoprotection after treatment with the composite of Pien Tze Huang. For example, after CCL₄ injury and treatment by PTH, the transaminase level went down 40% and the necrotic loci disappeared [1]. The composite of PTH contained four major components which were musk, Calculus bovis, snake gall and the root of Panax notoginseng.

High-performance liquid chromatography (HPLC) analysis at 204nm revealed at least four prominent peaks in the extract [1]. In this work, we further explored the potential of this old traditional composite via the use of mice model which had bile duct ligation or acute alcohol treatment. The methods entailed histopathological observation, biochemical techniques and histochemical techniques.

Bile duct ligation, performed by experienced investigators, was frequently used as models to address problems of obstructive cholestasis. In the neonatal, obstructive cholestasis if untreated is often quickly fatal. Surgical treatment often necessitates the construction of bypass to the intestine from the bile duct. In experimental animals, ligation of bile duct led to inflammatory changes followed by fibrosis [2]. In a later study [3], the same group reported severe inflammation before seven days after ligation and fibrosis started from three weeks postsurgery. The fibrosis usually began from the portal area and moved centrally; its formation involved stellate cells which differentiated into myofibroblasts and which then formed new collagen [2, 3]. Hyperplasia of bile ducts was also a feature and downregulation of histidine decarboxylase or VEGF could prevent this type of hyperplasia [4]. On the other hand, it has long been known that acute alcohol induced lipogenesis resulting in a fatty liver and long and chronic consumption of alcohol led to cirrhosis [5, 6] and the intensity of the damage appeared to be related to the percentage of the alcohol in the drinks [7]. Alcohol had also been injected into the liver to perform ablation to the tumor tissues [8]. Not only oral consumption of alcohol would give similar changes [9] In the first part of our experiment, we would compare a number of marker changes in the livers of two animal models, one of which had common bile duct ligation for 7 days and another had acute alcohol

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treatment for 7 days (30% ethanol, 10ml/kg/day intraperitoneally). Subsequently we would study four major Chinese herbs which acted together for treating liver ailment for more than 500 years in China and Eastern Asia in the composite name of Pien Tze Huang and evaluate the possible evidence in treatment of two animal disease models. These constituents were Calculus bovis, Mochus, Snake gall and Notoginseng radix.

2. Materials and Methods

2.1 Experimental and control mouse groups.

ICR young adult (12-week old) male mice were randomly divided into five groups, namely Control, Alcohol+saline, Alcohol+4 herbs, BDL+saline, BDL+4 herbs. The Control (n=7) group had no treatment. Both Alcohol+saline (n=7) and Alcohol+4 herbs (n=7) groups received intraperitoneal acute alcohol injection (30% ethanol, 10ml/kg/day) for 7 days. On the same day, Alcohol+saline group was intragastrically infused with 0.9% saline daily for 7 days as treatment control and Alcohol+4 herbs group was intragastrically infused with a formula containing Calculus bovis, Moschus, Snake gall, Notoginseng radix (370mg/kg/day, in the proportion of 1:1:1:1) for 7 days. On the other hand, both BDL+saline (n=7) and BDL+4 herbs (n=7) groups underwent bile duct ligation (BDL) procedure. After three days of recovery, BDL+saline group was intragastrically infused with 0.9% saline daily for 7 days as treatment control and BDL+4 herbs group was intragastrically infused with a formula containing Calculus bovis, Moschus, Snake gall, Notoginseng radix (370mg/kg/day) for 7 days.

2.2 Ligation of bile duct

The common bile ducts were ligated. All equipment and tools (forceps, scissors, clamps, needles, knives, cotton, gauge and threads) were sterilized. The mice were anesthetized by intra-peritoneal injection with 10% chloral hydrate (3mg/kg) and placed in supine position. The skin was disinfected with 70% ethanol. A cut (1cm) was made along the abdomen below the right costal margin and subcutaneous tissue was separated to expose the bile duct. The bile duct was then ligated with 10-0 suture. After suturing the skin with 4-0 suture, penicillin and Temgesic (Buprenorphine) were given. The mice were returned to the cage when they could move and showed no abnormal behavior. All procedures received institutional ethical approval. Out of the 7 animals for each group, 3 were used for transaminase biochemistry and 4 were used for histopathology and immunohistochemistry (including TUNEL, in situ method).

2.3 Histomorphology and Immunohistochemistry

Mice in all groups were killed and livers were dissected out and fixed and processed to paraffin blocks after alcohol dehydration and xylene clearance. Liver tissue was sectioned at 5 um thick and stained with hematoxylin and eosin for routine observation. For immunohistochemistry, sections were deparaffinized and rehydrated. The sections were first washed with 0.05% tween-20 in phosphate buffer saline (PBST) twice of 5 min each. Then the sections were heated in citrate buffer (10 mM, pH6.0) at 100°C for 15 min before they were treated with 3% hydrogen peroxide for 10 min.

After that, the sections were washed with PBST and blocked in 2% (w/v) bovine serum albumin (BSA) (Albumin, Bovine, 10857, USB Corporation, OH) for 1hour. After rinsing three times with PBST, the sections were incubated with one of the following primary antibodies overnight at 4°C (anti-proliferating cell nuclear antigen (PCNA) antibody (dilution: 1:1000; ab29, Abcam, Cambridge, UK), anti-transforming growth factor beta (TGF-beta) antibody (dilution: 1:1000; ab66043 Abcam, Cambridge, UK) and anti-lactate dehydrogenase (LDH) antibody (dilution: 1:1000; ab52488, Abcam, Cambridge, UK). On the following day, after washing with PBST three times, the sections were incubated with biotin conjugated goat anti-mouse secondary antibody (dilution: 1:1000; 31800, Invitrogen, CA) for PCNA and biotin conjugated goat anti-rabbit antibody (dilution: 1:1000; 65-6140, Invitrogen, CA) for TGF-beta and LDH in 2% (w/v) BSA for 2 hour to room temperature. After washing with PBST three times, the sections were incubated with streptavidin-horseradish peroxidase (HRP) conjugate (dilution: 1:500; 43432, Invitrogen, CA) in PBST for 2 h at room temperature. Washed with PBST twice again, these sections were incubated in 5% 3, 3-diaminobenzidine (DAB) for 5–10 min until brown colors developed. PCNA labeling was used for visualization of proliferative nuclei and LDH labeling served as a marker for necrosis. For quantitation, the PCNA positive nuclei were counted in 12 randomly selected areas (size 700 um² per area) from all specimens (n=3 fields for each animals) of 4 animals of each group. The TGF-beta, LDH activities were quantified via positive cell count and number of positive patches greater than 200μm², respectively. Again 12 random fields were selected from 4 animals of each group. The mean and standard deviations were compared between groups and P < 0.05 was considered significant.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to analysis the degree of apoptosis of cells, and was performed on liver sections. For the TUNEL study, ApopTag1 Peroxidase In situ Apoptosis Detection Kit (Millipore Corporation, Billerica, MA) was used. According to the manufacturer’s instruction, liver sections were first deparaffinized with xylene, rehydrated and treated with proteinase K (20 ug/mL) for 15 min at room temperature. After quenching with 3.0% hydrogen peroxide, the sections were treated with biotin-deoxyuridin triphosphate in the working solution of deoxynucleotidyl transferase (TDT) for 1 hour in a humidified chamber at 37°C.
The reaction was stopped with stop/wash buffer for 10 min at room temperature. To detect the binding of dioxigenin-11-dUTP, sections were then treated with the anti-digoxigenin conjugate for 30 min at room temperature. Finally, after visualization with 3,3-dimethylaminoazobenzene (DAB), apoptotic cells with cellular DNA fragmentation were photographed with a digital camera (Zeiss Axioscam MRC5). For the negative controls, the TdT enzyme was substituted in deionized water with the same procedure, while positive controls were prepared by incubating the pretreated sections with DNase I (dilution: 1.0ug/mL, DN25, Sigma-Aldrich, St. Louis, US) for 10 min at room temperature.

For histopathology, slides of livers from control and experimental groups were stained with Sirius red (Merck Chemicals, Darmstadt, Germany) for 1 h and further washed in 2 changes of acidified water to visualize collagen fibers of connective tissue. After this, the slides were further dehydrated in 3 changes of 100% ethanol, cleared in xylene and mounted in Permount. The relative percentage of Sirius red positive areas per total area in each field of 3000µm² were calculated; 12 fields were taken from 4 animals of each group.

2.4 Aspartate aminotransferase (AST) activity assay

Serum was collected and aspartate aminotransferase (AST) activities were quantified in order to observe the damage of the liver tissue under different circumstances. The AST activity assay kit (MAK055, Sigma-Aldrich, St. Louis, US) and ALT activity assay kit (MAK052, Sigma-Aldrich, St. Louis, US) were used. Liver tissue was first homogenized in respective assay buffer and centrifuged in order to remove insoluble materials. Then, samples were loaded in to a 96 well-plate and mixed with the master mixed solution provided in the assay kit and incubated in 37°C for 5 minutes and samples absorbance were measured at 450nm for the AST activity assay kit and 570nm for ALT activity assay kit.

3. Results

AST activities quantitation revealed that both alcohol treatment and common bile duct ligation induced increase amounts of AST activities after alcohol treatments of 7 days and bile duct ligation of 10 days (including three days for recovery after ligation) (Figs. 1a & b). For the animals treated with these four herbs along with either bile ligation or acute alcohol treatment, the AST activities levels decreased, though still at a higher level than that of control (Fig. 1a & b). Sirius red staining for collagen fiber indicated increased collagen amount after bile ligation, starting from the portal triad and invading into the central liver (Fig. 2a). For those animals treated with the four Chinese herbs which had bile duct ligation, the liver had less collagen fiber, in spite of the presence of necrotic centers (Fig. 2b). For the animals treated with acute alcohol intraperitoneally, with and without Chinese medicine treatment, there was no intensive fibrosis after one week of alcohol (Fig. 2c). The percentage of collagen positive areas versus the total fields in all groups were depicted in Figure 3. There was evidence that collagen formation was less in the two experimental groups after addition of the four herbs.

For studies in PCNA histochemically, the animals with biliary ligation and without drug treatment had less positive nuclei while those that had ligation and treated with the four herbs had more proliferative nuclei (Fig. 4a & b). For the alcohol treatment group, increased proliferative nuclei were also apparent in the experimental group with drug treatment (Figs. 4b & c). Quantitative comparison of the groups in the density of proliferative nuclei was depicted in Figure 5.

Studies on the TGF-beta positive sites revealed positive sites spreading all through the liver in the animals with bile duct ligation whilst in the animals with bile duct ligation and drug treatment, some areas of the liver had no positive TGF-beta sites (Figs. 6a & b). On the other hand, acute alcohol treatment demonstrated very few TGF-beta positive sites in both alcohol groups with and without drugs treatment (Fig. 6b). Quantitation of TGF-beta positive site per unit area in the four groups were depicted in Figure 7. For detection of necrosis employing LDH, immunocytochemistry depicted positive sites surrounding and not far away from bile ducts while for those animals that had ligation and 4 herbs treatment, there was no positive necrotic cells (Fig. 8a & b). In the acute alcohol model, there was necrotic focus in every lobule (Fig. 8c) which was not observed in the acute alcohol model with drug treatment (Fig. 8d). Quantitation on the necrotic patches of the size larger than 200µm² of all groups were depicted in figure 9. TUNEL studies on apoptotic cells were few or absent in both biliary ligation group with and without additional drug treatment (Fig. 10a & b) but there were a significant number of TUNEL positive cells in the alcohol model which had decreased in the alcohol model with drug treatment (Fig. 10c & d). A quantitation of the TUNEL positive cells per unit area (700µm²) of all groups was depicted in Figure 11.

4. Discussion

Bile duct ligation in the animals had been reported to cause significant damage in the liver of rodent models, which included increase of transaminase and cell death [10, 11]. Upregulation of blood bilirubin was most prominent by the 5th day of ligation and was still higher than the normal 15 days after [12]. Collagen formation in ligated mice reached its peak by three weeks after ligation [13]. Our picture of the ligation fits into the general picture of fibrosis except that collagen formation in our models presented itself very early after ligation. On the other hand, collagen formation was not a feature of acute alcohol intoxication (present studies), unlike those of long term chronic alcohol uptake. There
were obvious differences between these two animal models of bile duct ligation and acute alcohol treatment. For the biliary ligation model, there were substantial fibrosis, more TGF-beta positive sites and more necrotic sites. On the other hand, acute high percentage of alcohol for short term featured more apoptosis with little fibrosis. The livers of both models improved after the combination treatment of the four Chinese herbs in terms of fibrosis, cell death and cell proliferation indicating these drugs acting together had indeed potential therapeutic effects on the liver as far as animal models were concerned. These four herbs were selected as they were prominent components of liver protection and therapeutic remedies for centuries in China. Calculus bovis, for example, was known for its anti-inflammatory, antipyretic, neuroprotective and antispasmodic effects [14-16]. There were oxysterols inside this component and one of its constituents taurine protected tissue against high concentration of calcium [17], thus exerting an impact on cell death.

The second component was the Moschus, containing extracts of preputial glands of male musk deer. Experiments on Moschus indicated that this too contained steroids which were antiapoptotic, anti-inflammatory and had androgenic and cardiovascular stimulatory activities [18, 19]. The third drug was the snake gallbladder which had few studies. One of the most important was from a Japanese group which indicated that the alcoholic extract of the snake gallbladder increased the phagocytosis of peritoneal macrophages and their lysosomal enzyme production [20], which dealt with the defense and perhaps protected from cell death. The last drug of the list was the Notoginseng radix which had been studied immensely in recent literature. It contained saponins and ginsenosides and has been found to protect against fibrosis, cell death, suppressed and inhibited calcium elevation and superoxide formation and protected against oxygen and glucose deprivation [21-23]. Other studies showed that Notoginseng could lower serum triglyceride via the increase of PPAR alpha mRNA which could combat the effect of chronic alcohol consumption [24]. On the whole, it appeared that protection from cell death, antivasular spasm and antistress were prime targets of the Calculus bovis, Moschus and Notoginseng radix were anti-inflammatory agents which were mainly effects (of) Calculus bovis and Moschus. For antifibrosis, the primary agent appeared to be Notoginseng radix [21-24]. It appears this study strengthens the mechanistic background and therapeutic value of the major components of the centuries old remedy does have hepatoprotection effects in animal models.

**Fig. 1a, b.** Decrease in AST levels after herbal treatment in mice that had undergone bile duct ligation (BDL) (P < 0.01, BDL+4 herbs vs. BDL+saline, Newman-Keuls test) or acute alcohol treatment (P<0.05, Alcohol_4 herbs vs. Alcohol+saline, Newman-Keuls test).

**Fig. 2a.** For mice undergone bile duct ligation, Sirius red staining for collagen fiber indicated increased collagen amount, starting from the portal triad and invading into the central liver

**Fig. 2b.** For the BDL animals treated with the four Chinese herbs, the liver showed less collagen fiber, in spite of the presence of necrotic centers

**Fig. 2c.** For the animals treated with acute alcohol intraperitoneally, with the Chinese medicine treatment, there was no intensive fibrosis after one week of alcohol
Fig. 3. The percentage of collagen positive areas versus the total areas were depicted. There was evidence that collagen formation was less in the two experimental groups after addition of the four herbs ($P<0.0001$, BDL+4 herbs vs. BDL+saline, Student’s t test; $P<0.0001$, Alcohol+4 herbs vs. Alcohol+saline, Student’s t test).

Fig. 4a. For studies in proliferative nuclear antigen histochemically, the animals with biliary ligation and without drug treatment had less positive nuclei while those that had ligation and treated with the four herbs had more proliferative nuclei.

Fig. 4b. For the alcohol treatment group, increased proliferative nuclei was also apparent in the experimental group with drug treatment.

Fig. 5. Quantitative comparison of the groups in the density of proliferative nuclei was depicted ($P<0.0001$, BDL+4 herbs vs. BDL+saline, Student’s t test; $P<0.0001$, Alcohol+4 herbs vs. Alcohol+saline, Student’s t test).
Fig. 6a. Studies on the TGF-beta positive sites revealed positive sites (A) spreading all through the liver in the animals with bile duct ligation whilst in the animals with bile duct ligation and herbs, some areas of the liver had no positive TGF-beta sites (B).

Fig. 6b. Acute alcohol treatment demonstrated very few TGF-beta positive sites in both alcohol groups with and without other drug treatment.

Fig. 7. Quantitation of TGF-beta site per unit area in the four groups were depicted (P<0.0001, BDL+4herbs vs. BDL+saline, Student’s t test).

Fig. 8a. For detection of necrosis employing lactic dehydrogenase, immunocytochemistry depicted positive sites surrounding and not far away from bile ducts (arrow) while those animals that had ligation and drug treatment, there was no positive necrotic cells.

Fig. 8b. In the acute alcohol model, there was necrotic focus in every lobule (arrow) which was not observed in the acute alcohol model with drug treatment.

Fig. 9. Quantitation on the necrotic patches of the size larger than 200um2 of all groups were depicted (P<0.0001, BDL+4herbs vs. BDL+saline, Student’s t test; P<0.0001, Alcohol+4herbs vs. Alcohol+saline, Student’s t test).
Fig. 10a. TUNEL studies on apoptotic cells were few (arrow) in both biliary ligation group with and without additional drug treatment.

Fig. 10b. but there were significant number of TUNEL positive cells (arrows) in the alcohol model which had decreased in the alcohol model with drug treatment.

Fig. 11. A quantitation of the TUNEL positive cells of all groups was depicted (P<0.0001, BDL+4herbs vs. BDL+saline, Student’s t test; P<0.0001, Alcohol+4 herbs vs. Alcohol+saline, Student’s t test).

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