Evaluation Protective Effect of Silymarin Against Carbon Tetrachloride-induced Liver Injuries in Rats and Mice Models

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Background: Bloodstream infection (BSI) is an important cause of serious morbidity and mortality for hospitalized patients. Empirically Gram stain of bacteria gives the first clue for the etiology of infection and medical treatment. But the delayed treatment on 1 or 2 days after phenotypic identification and drug susceptibility testing may cause potential danger to patients. Rapid drug susceptibility testing can provide earlier information to guide treatment and in less time than bacterial culture and sensitivity testing, for antibiotics therapy.

Methods: In this study, we excluded samples of polymicrobial bacteremia. We collected isolates from 815 infection episodes caused by Escherichia coli (57%), Klebsiella pneumoniae (20.16%), Enterobacter cloacae (6%), P. aeruginosa (9.1%), Stenotrophomonas maltophilia (3.1%), and Acinetobacter baumannii (3.1%) in a 10-month period. We identified those bacteria with direct susceptibility test with the use of Phoenix100 (BD) during a 10-month period.

Results: The results of direct susceptibility were concordant (99%-100%) with those obtained from Phoenix100.

Conclusion: These results have the potential to guide clinicians to initiate an early antimicrobial therapy in febrile patients with sepsis shock.

Key words: bloodstream infection, bacteremia, direct susceptibility test

Introduction

A wide variety of phytochemicals inclusive of Silymarin have been reported to have substantial anti-carcinogenic activity because of their antioxidant and antiinflammatory properties. Silymarin, a flavonolignan, extracted from the fruits and seeds of the plant milk thistle (Silybum marianum L. Gaertn.)(1). Milk thistle belongs to the family of Asteraceae and primarily is an indigenous plant of Mediterranean region and southwest Europe. Silymarin is a mixture of mainly three flavonolignans, Silybin (Silibinin), Silydianin and Silychristin [2-3]. Silibinin is the major (70–80%) and most active biological component of Silymarin. The seeds of milk thistle have been used for the last 2,000 years for liver diseases. Pharmacological studies revealed that Silymarin is non-toxic even at higher physiological doses, which suggests its safer use for humans [4]. Laboratory studies suggest that there is no significant difference between Silymarin and Silibinin in
terms of chemopreventive or biological activities conducted in several in vitro and in vivo cancer models. Silymarin has been primarily used in liver disorders including hepatitis, alcoholic liver diseases and cirrhosis and is also useful for toxin-induced liver toxicity, including poisoning from a fungus called death cap mushroom (Amanita phalloides). Although its clinical efficacy is currently uncertain, interest in this botanical medicine has been piqued by studies showing Silymarin blocks HCV cell culture (HCVcc) infection. Intravenous administration of Silibinin, composed of a 1:1 mixture of Silybin A and Silybin B, causes dose-dependent reduction of viral load in patients with chronic hepatitis C. Topical treatment of Silymarin inhibited photocarcinogenesis in mice in terms of tumor incidence, tumor multiplicity and growth of the tumors. Wide range of in vivo mechanistic studies conducted in variety of mouse models indicated that Silymarin has anti-oxidant, anti-inflammatory and immunomodulatory properties which led to the prevention of photocarcinogenesis in mice. Based on the anti-oxidant and anti-inflammatory activity of Silymarin, the chemopreventive effect of Silymarin has been tested and determined using animal models of photocarcinogenesis.

Since then extensive mechanism-based chemopreventive studies have been performed in vitro in cell culture, we performed in vivo animal models to assess the efficacy of Silymarin. The purpose of this research is to examine and compare biological activities of Silymarin in rats and mice with chronic CCl₄-induced liver injury, to evaluate the therapeutic effects of Silymarin, to figure out whether therapeutic effect was in agreement with in vitro.

Materials and Methods

Reagents

Carbon tetrachloride (CCl₄), olive oil and other reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Diagnostic kits for assaying alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Arkray, Inc. (Kyoto, Japan). Other assay kits were obtained from Cayman Chemical (Ann Arbor, MI, USA).

Animals

Male Wistar rats and BALB/c mice, specific pathogen-free, 6 weeks old and weighing 250-300 g and 20-25 g individually, obtained from the Animal Medicine Center, College of Medicine, National Taiwan University were used for the chronic CCl₄-induced liver injury model in all experiments. The animals were maintained in a standard laboratory under a 12-h light/dark cycle in a temperature (20 ± 2 °C), humidity (75 ± 15%), and filtered laminar air flow controlled room in the animal facility of the Animal Medicine Center, College of Medicine, National Taiwan University, Taipei, Taiwan. Rats were raised and cared for given autoclaved water and fed by ad libitum with laboratory pellet chow following the guidelines set up the National Science Council of the Republic of China. Experiments were performed according to law, regulations and guidelines for animal experiments in Taiwan, which are in agreement with the Helsinki declaration.

Experimental design

Thirty rats were divided into three groups (each group consisted 10 rats). Thirty mice were also divided into three groups (each group consisted 10 mice). (1) As a normal control, animals were fed with regular diet and double distilled water. (2) Animals were treated with 40% CCl₄/olive oil (1 mL/kg body weight per day, i.p. twice per week) for 8 weeks to induce chronic chemical liver injury as a negative control. (3) Animals were treated with 40% CCl₄/olive oil (1 mL/kg body weight per day, i.p. twice per week) and with Silymarin (11.667mg/0.3cc/rat or 3.889/0.1cc/mice, p.o., 4 days per week) treatment for 8 weeks. Blood samples were collected (0.2 mL with 10 U/mL heparin) in orbital bleeding at the end of the first, third, sixth, and eighth weeks. At the end of the experiments, blood and livers were collected immediately after the animals were sacrificed at week 8 under anesthesia by CO₂. Livers were weighed and utilized for the following biochemical and histology analyses.

Measurement of plasma transaminase activities

Plasma samples were prepared at the end of the eighth weeks and analyzed using ALT and AST diagnostic kits, following the manufacturer’s protocols. ALT and AST enzyme activities were measured by Hitachi 717 (Tokyo, Japan) biochemical instrument and Boehringer Mann-
Glutathione peroxidase assay

Liver was homogenized with GSHPx cold buffer (50 mM Tris-HCl containing 5 mM EDTA and 1 mM di-thiothreitol (DTT), pH 7.5). GSHPx activity was measured by a GSHPx assay kit [16]. The reaction was initiated by the mixing glutathione, glutathione reductase, NADPH with cumene NADPH to NADP with a spectrophotometer at 340 nm for 5 min. The specific activity measured as nanomoles of NADPH oxidized to NADP per minute per milligram protein.

Catalase assays

Liver tissues were homogenized in a cold buffer (50 mM potassium phosphate and 1 mM EDTA, pH7.0). Supernatant was collected and added with hydrogen peroxide as exogenous substrate. The activity of catalase was measured using a catalase assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) as reported previously [18]. Change in the absorbance was monitored at 540 nm by according to the manufacturer's protocol.

Histology assay

Rats and mice were sacrificed at the end of the eighth week, and the livers were immediately removed. Liver slices were made from a part of the left and central lobes, and immediately fixed in 10% buffered formalin phosphate solution, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Liver pathology was rated on four levels based on Ruwart et al. [19]: None (I), no detectable pathological alternation; focal (II), focal and local alternation; multifocal (III), multiple focal alterations; Diffuse (IV) represents bread diffuse alteration. Stage assessment of hepatic fibrosis was based on Jon less: (I), fibrosis only occurring in the portal area; (II), portal area fibrosis extends to the hepatic lobule but focal fibrosis does not link these; (III), the portal fibrosis extends into the hepatic lobule, a clear pseudo-lobule is formed and bile duct hyperplasia occurs.

Statistical analysis

The experimental results are expressed as the means ± SEM. Data were assessed by analysis of Student’s t-test. P<0.05 was considered as the level of significance between silymarin treatment group and CCl₄ induction group.

Results

Plasma Transaminase

Blood was collected at the indicated times for AST and ALT determination after CCl₄ administration for rats and mice. Both plasma AST and ALT levels were markedly increased (table 1) after carbon tetrachloride treatment as compared the normal group (double distilled water treatment). For rats and mice, serum ALT levels were 64.00±9.66 IU/L and 75.67±26.94 separately and AST levels were 148.00±18.74 IU/L and 340.13±121.15 individually at the end of the eighth weeks. On the other hand, Silymarin did not significantly decreased ALT and AST levels of rats and mice at the eighth week as compared with those of the CCl₄ group (P>0.05).

Assessment of antioxidant enzyme activities

To investigate the role of anti-oxidant enzyme activities in the liver during CCl₄ injury, we analyzed catalase CCl₄ toxicity resulted in lower catalase for rats (15.84±5.98) but not mice (13.19±5.65). We also evaluated GPx activity and found lower GPx activity for rats but not mice (table 1). Silymarin administrations do not significantly alter catalase and GPx activity caused by CCl₄ effect (table 1).

Histopathological assessment

Liver tissue sections of rats and mice were stained with H&E (Figure 1 and 2). Negative control tissue sections exhibited no apparent pathological alternations (Figure 1A and 2A). No cavitations, necrosis or fibrosis were found in control sections. In contrast, sections from CCl₄-only treated rats and mice displayed cavitations and fibrosis on broad areas (Figure 1B and 2B). The broad cavitations and fibrosis in livers were attenuated in rats and mice treated with Silymarin during the experimental periods. Silymarin administration for rats and mice did result in less cavitations and fibrosis in the liver.
Silymarin treatment also elevated the survival rate of rats and mice, especially the rats induced by CCl₄. We also examined the distribution of cavity and fibrosis in different liver regions (from central vein region to hepatic portal veins) for rats and mice shown by Table 2. Silymarin reduced apparent liver injury caused by CCl₄ for rats and mice by histopathological assessment.

### Table 1 Effects of the Silymarin on plasma ALT, AST, catalase and GPx in CCl₄-intoxicated rats and mice.

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>ALT</th>
<th>Catalase activity (nmol/min/mg protein)</th>
<th>GPx activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>148.00±18.74</td>
<td>64.00±9.66</td>
<td>29.78±7.48</td>
<td>66.34±27.13</td>
</tr>
<tr>
<td>CCl₄</td>
<td>1307.00±1230.24ᵃ</td>
<td>826.00±675.41ᵃ</td>
<td>15.84±5.98ᵇ</td>
<td>46.01±19.95ᶜ</td>
</tr>
<tr>
<td>Silymarin</td>
<td>1638.89±950.91ᵇ</td>
<td>1298.89±841.27ᵇ</td>
<td>12.83±5.15ⁱᵇ</td>
<td>55.10±12.14ᵇᵇ</td>
</tr>
<tr>
<td>Normal</td>
<td>340.13±121.15</td>
<td>75.67±26.94</td>
<td>10.14±3.70</td>
<td>57.63±9.81</td>
</tr>
<tr>
<td>CCl₄</td>
<td>1137.33±664.02ᵃ</td>
<td>785.44±404.02ᵃ</td>
<td>13.19±5.65ᶜ</td>
<td>49.57±12.20ᵃ</td>
</tr>
<tr>
<td>Silymarin</td>
<td>1430.00±694.26ᵇᵇ</td>
<td>1220.00±701.89ᵇᵇ</td>
<td>9.81±5.42ᵈᵇᵇ</td>
<td>48.07±11.26ᵈᵇᵇ</td>
</tr>
</tbody>
</table>

a: Silymarin therapy or CCl₄-induction group significantly increased the level of AST or ALT compared with normal group. (p<0.05)
b: Silymarin therapy group was not significantly effective to reduce the level of AST or ALT compared with CCl₄-induction group. (p>0.05)
c: Silymarin therapy or CCl₄-induction group significantly decreased the level of catalase or GPx activity compared with normal group. (p<0.05)
d: Silymarin therapy group was not significantly effective to increase the level of catalase or GPx activity compared with CCl₄-induction group. (p>0.05)
e: Silymarin therapy group or CCl₄-induction group was not significantly different with normal group. (p>0.05)

(Figure 1C to 1D and 2C to 2D). Silymarin treatment also elevated the survival rate of rats and mice, especially the rats induced by CCl₄. We also examined the distribution of cavity and fibrosis in different liver regions (from central vein region to hepatic portal veins) for rats and mice shown by Table 2. Silymarin reduced apparent liver injury caused by CCl₄ for rats and mice by histopathological assessment.

**Conclusion**

Carbon tetrachloride has been widely used to induce liver injury in animal models [20]. CCl₄ is metabolized to CCl₃ by CYP450 in hepatocytes. Subsequently, CCl₃ reacts with oxygen to form CCl₃OO. Both CCl₃ and
CCl₃OO trigger super-oxidative chain reactions in lipid molecules [21]. Further reactions with phospholipids and polyunsaturated fatty acids can affect the permeability of the cell membrane, the mitochondria, and the reticular membranes [22]. Consequently, elevation of cytosol calcium leads to cell damage [23]. The results in this experiment show that both plasma ALT and AST levels were markedly increased after carbon tetrachloride treatment as compared the normal group (double distilled water treatment). It also means that the experiment is successful to induce liver injury.

Medicinal nutrients derived from plants have been used for health maintenance and disease management since the dawn of history. One class of phytomedicines currently receiving increased scrutiny is the polyphenols. These number in the thousands and include, but are not limited to, the various flavonoid subclasses. Plant-derived polyphenols are increasingly receiving attention as dietary supplements for the homeostatic management of inflammation, to support detoxification, and for anticancer, weight loss, and other benefits. Their pro-homeostatic effects on genes, transcription factors, enzymes, and cell signaling pathways are being intensively explored, but the poor bioavailability of some polyphenols likely contributes to poor clinical trial outcomes. Many polyphenols are very poorly absorbed when taken orally, posing the greatest obstacle to routine clinical application [24]. Where possible, the conversion of polyphenols to phytosome forms improves oral bioavailability without compromising safety.

Silybin with the polyphenol structure is an effective antioxidant, conserving glutathione (GSH) in liver cells while stabilizing the liver cell membranes against oxidative attack [25]. Its antioxidant potency is bolstered by its effective chelation of iron. In fact, in a human clinical trial silybin even lowered serum ferritin [26]. Silybin is a proven liver protectant; in animal experiments it blocked the oxidative toxicities of acetaminophen, alcohol, carbon tetrachloride, and the mushroom toxins phalloidin and alpha-amanitin [27]. These findings correlate with decades of clinical observations that silybin improves survival for humans exposed to deathcap mushrooms (Amanita species) [28].

After Silymarin treatment, the results showed that ALT and AST levels were not decreased for rats and mice. (P>0.05) Silymarin administrations do not alter catalase and GPx activity caused by CCl₄ effect. In our CCl₄-induced chronic liver injury model, we observed that the livers in Silymarin treated rats and mice displayed less injury in the histochemical analyses compared to those in the CCl₄-only treated group. Hepatic fibrosis, necrosis, and cavitations caused by CCl₄ were attenuated by Silymarin administration. These results indicate that Silymarin has recovery/reparative effects on CCl₄-induced liver injury. However, comparison of the data of transaminase and histopathological assessment is very difficult and should be cautious on account of significantly disagreement of administration results. If we judge the results only by transaminase administration, we may not conclude that Silymarin is able to cure chemical liver injury in Rats and mice. This conclusion is not in agreement with histopathological assessment.

In this study, we also find that Silymarin treatment is not so effective to cure chemical liver injury in rats and mice (table 2). After 8 weeks treatment, there are still 7 rats and 4 mice to display cavitations on level II and III. Tissue sections also exhibit poor fibrosis. Transaminase elevates at the beginning of liver injury. I am of the opinion that cell structures returned to some recovery but not to fully normal healthy state and it caused abnormal levels of transaminase we observed. We should try to find some other medicine in the coming future to instead of Silymarin.

Of particular importance, we suggested that rat model is superior to mouse model because cavitations and fibrosis on broad areas are more serious while liver sections treated by CCl₄ from rat model. Sections from CCl₄-only treated mice displayed not so apparent cavitations and fibrosis on broad areas compared with those of rats model. We may say for sure that rat model can sub-

<table>
<thead>
<tr>
<th>Rats</th>
<th>Level of cavitation</th>
<th>Level of fibrosis</th>
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<tr>
<td></td>
<td>Total</td>
<td>I</td>
</tr>
<tr>
<td>Negative control</td>
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<td>0</td>
</tr>
<tr>
<td>CCl₄ control</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Silymarin treatment</td>
<td>10</td>
<td>3</td>
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<table>
<thead>
<tr>
<th>Mice</th>
<th>Level of cavitation</th>
<th>Level of fibrosis</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>I</td>
</tr>
<tr>
<td>Negative control</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>CCl₄ control</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Silymarin treatment</td>
<td>10</td>
<td>6</td>
</tr>
</tbody>
</table>
stitute for mouse model to evaluate medicine treatment effect.

References


14. Agarwal R, Katiyar SK, Lundgren DW, Mukhtar H. Inhibitory effect of silymarin, an anti-hepatotoxic flavonoid, on 12-O-tetradecanoylphorbol-13-acetate-induced epi-


評估水飛薊素保護四氯化碳所導致大、小鼠肝損傷之效用模式

吳銘芳 1  葉明陽 2  許佑銘 1  唐明珠 1  陳雪琴 1  李青松 5  薛樹清 3  鍾明燈 4  陳有任 6  吳旭峰 3,5,6

1 台灣大學動物中心
2 副院長室
3 臨床病理科
4 藥物治療
5 輔仁大學民生學院餐旅管理系
6 元培科技大學醫學檢驗生物技術系

水飛薊素(Silymarin)從奶薊中提煉而出，長久以來一直是歐洲治療肝病的傳統良方。本研究以四氯化碳導致雄性Wistar大鼠與BALB/c小鼠肝損傷模式，評估水飛薊素治療效果。以大鼠11.667 mg/0.3cc與小鼠3.889 mg/0.1cc之水飛薊素每週治療四次，不論是大或小鼠(每組10隻)其血清中的AST與ALT皆未因治療而有所改善。此外經由水飛薊素治療的大、小鼠其穀胱甘肽過氧化酶(Glutathione peroxidase)與過氧化氫酶(catalase)皆未改善。令人好奇的是病理組織卻發現以水飛薊素治療的大、小鼠肝損傷具明顯的病況改善，此種病理結果與血清化學檢查並不一致，可能因肝細胞構造雖然有些恢復，但尚未臻至正常健康狀態，因而血清指數仍異常。值得一提的是大鼠模式較優於小鼠模式，主因四氯化碳導致大鼠肝空洞化與纖維化較小鼠明顯。吾輩認為欲評估水飛薊素治療肝損傷大鼠優於小鼠。

關鍵詞：血液感染(bloodstream infection)、菌血症(bacterimia)、藥敏(susceptibility)