Expression of VDAC Regulated by Extracts of *Limonium sinense* Ktze root Against CCl₄-induced Liver Damage

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Abstract: The expression of mitochondrial voltage-dependent anion channels (VDAC) may underlie the protective effects of *Limonium sinense* (Girard) Ktze root extracts (LSE) against carbon tetrachloride-induced liver damage. Pretreatment of mice with 100 mg/kg, 200 mg/kg or 400 mg/kg LSE significantly blocked the carbon tetrachloride-induced increase in both serum aspartate aminotransferase (sAST) and serum alanine aminotransferase (sALT) levels. Ultrastructural observations by electron microscope confirmed hepatoprotection, showing decreased nuclear condensation, ameliorated mitochondrial fragmentation of the cristae and less lipid deposition. Pretreatment with LSE prevented the decrease of the disruption of mitochondrial membrane potential (15.3%) observed in the liver of the carbon tetrachloride-insulted mice, further demonstrating the mitochondrial protection. In addition, LSE treatment (100-400 mg/kg) significantly increased both transcription and translation of VDAC. The above data suggests that LSE mitigates the damage to liver mitochondria induced by carbon tetrachloride, possibly through regulation of mitochondrial VDAC, one of the most important proteins in the mitochondrial outer membrane.

Keywords: Limonium sinense (Girard) Ktze, liver, mitochondria, voltage-dependent anion channels

1. Introduction

Evidence has accumulated that cell death is involved in liver injury and liver disease. Apoptosis and necrosis underlie many types of liver injury, including fibrosis, alcoholic liver disease and hepatitis [1, 2]. Mitochondria play a key role in controlling cell death, not only providing ATP by oxidative phosphorylation but also modulating intracellular Ca^{2+} homeostasis and pH, and induction of apoptotic and excitotoxic cell death [3]. Indeed, mitochondrial dysfunction contributes to a great number of human and animal diseases. Changes such as disruption in mitochondrial membrane potential occur in the process of liver injury and drugs could protect liver mitochondrial through preventing the dissipation of mitochondrial membrane potential in hepatotoxicated mice [4, 5, 6].

In the outer membrane of mitochondria, the voltage-dependent anion channel (VDAC) is a key protein that regulates basic mitochondrial functions such as energy transduction, substance metabolism, and intracellular calcium homeostasis as well as initiation of apoptosis via release of intermembrane space proteins [7]. Our previous studies have shown that both transcription and translation of liver VDAC changed significantly and accompanied the mitochondrial damage in liver damaged mice, which could be prevented by natural products [8].

Limonium sinense (Girard) Ktze is a folk medicine popularly used as a remedy for bleeding, piles, fever, hepatitis, diarrhea, bronchitis and other disorders [9]. The plant is mainly distributed along seashores and salts marshes in southern China, Ryukyus (Japan) and western Taiwan. Recently, *Limonium sinense* was demonstrated to possess hepatoprotective action against carbon tetrachloride (CCl_4) and D-galactosamine (D-GalN) intoxication in rats [10]. As reported by Lin et al.[11], the major constituents found in the leaves and the roots of *Limonium sinense* were flavonoids. However, the mechanisms underlying the antihepatotoxicity have not been investigated.

In the present study, we evaluated the hepatoprotective effect of *Limonium sinense* (Girard) Ktze (LSE) extracts against liver injury induced by CCl_4, addressing the possible action of LSE on liver mitochondrial and VDAC expression to search for the mechanism underlying its hepatoprotective activity.

2. Materials and Methods

2.1. Plant material

Roots of *Limonium sinense* were collected at the Yancheng Seabeach in China and identified by Mr. Yao Gan (Institute of Botany of Jiangsu Province, Chinese Academy of Sciences) in December 2005.

LSE was prepared as follows: dried cut roots of *Limonium sinense* (100 g) were extracted with water (800 ml) by reflux for two hours three times, and the extracts combined and subjected to evaporation to obtain 32.89 g (yield: 32.89 % w/w) of crude extract of *Limonium sinense* (LSE).

2.2. Chemicals

Rhodamine123 (Rh123), succinate, rotenone and anti-VDAC antibody were purchased from Sigma (St Louis, MO, USA). Tripure reagent was from Roche diagnostics corporation (Indianapolis, USA).

AMV reverse transcriptase, RNase inhibitor, dNTP, Oligo(dT)$_{15}$, and Taq polymerase were all from Promega. All other chemicals were of high purity from commercial sources.

2.3. Animals

Male ICR mice (Experiment Animal Center of Nanjing Medical University, Nanjing, P. R. China, Certificate No. SCXK 2002-0031) weighing 18-22 g were used. All animals were fed a standard diet *ad libitum* and housed at a temperature of 20-25°C under a 12-h light/dark cycle throughout the experiment. All animals received humane care and the study protocols complied with the guidelines of Nanjing University.

2.4. Carbon tetrachloride (CCl4)-induced hepatotoxicity in mice

Mice were randomly divided into five groups of eight animals each. All mice except the normal received 0.15% CCl$_4$ (in olive oil, 10 ml/kg, *i.p.*). The normal and CCl$_4$ groups received olive oil (10 ml/kg, *i.p.*) and CCl$_4$, following five days of oral treatment of saline. Drug groups were administrated with CCl$_4$, following five days of oral treatment of 100 mg/kg, 200 mg/kg, or 400 mg/kg LSE. Eighteen hours after the final treatment, blood was collected and mice were euthanized. The blood was allowed to clot at room temperature and serum was obtained by centrifugation at 3,000 g for 20 min. Meanwhile, the whole liver was excised and sections (~1 mm wide) were taken and fixed in a solution of 4% glutaraldehyde containing 3% paraformaldehyde and prepared for examination under electron microscope following standard techniques (JEM-1200EX). The remaining liver lobes intended for mRNA and protein analyses were frozen immediately and stored in liquid nitrogen before extraction.

2.5 Aminotransferase activity determination

Serum alanine aminotransferase (sALT) and aspartate aminotransferase (sAST) levels, markers for hepatotoxicity, were determined using an automatic analyzer (Hitachi 7600-020, Japan).

2.6 Isolation of liver mitochondria

Mitochondria were prepared from mouse livers according to the method of Apprille [12]. In brief, mouse livers were excised, homogenized in isolation buffer containing 225 mM D-mannitol, 75 mM sucrose, 0.05 mM EDTA and 10 mM Tris-HCl (pH 7.4) at 4°C. The homogenates were centrifuged at 600 *g* for 5 min and supernatants were centrifuged at 8,800 g for 10 min. The pellet was washed twice with the same buffer. Protein concentration was determined using Coomassie Brilliant Blue [13].

2.7. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi_m$) was evaluated according to Emaus et al. [14] by uptake of the fluorescent dye rhodamine 123 (Rh123), which accumulates electrophoretically into energized mitochondria in response to their negative inside membrane potential. Isolated liver mitochondria were suspended in the assay buffer (0.5 mg protein/ml) containing 225 mM mannitol, 70 mM sucrose, 5 mM HEPES(*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), pH 7.2. The

mitochondrial membrane potential ($\Delta\Psi_m$) was assessed spectrophotometrically (Hitachi 850) at 25°C with excitation at 505 nm and detection at 534 nm after addition of 0.3 µM Rh123. The membrane potential was calculated by the relationship: $\Delta\Psi_m$ =-59 log [Rh123]$_{in}$/[Rh123]$_{out}$, assuming that the distribution of Rh123 between mitochondria and medium follows the Nernst equation [15].

2.8 Evaluation of VDAC mRNA level by RT-PCR assay

Total RNA was extracted from livers using Tripure (Roche). Reverse transcription was started with 2 µg of total RNA at 42°C for 60 min in a 20 µl reaction mixture containing 20 U RNase inhibitor, 0.25 mM each of dNTP, 0.5 µg Oligo(dT)$_{15}$ and 15 U AMV reverse transcriptase. The reaction was terminated by incubation at 95°C for 5 min. PCR amplification was performed for 30 cycles, including 4 µl cDNA by adding 5 mM MgCl$_2$, 2.5 U Taq polymerase (Promega), 0.25 mM each dNTP, and 5'- and 3'-sequence-specific oligonucleotide primers for VDAC and β-actin in 1×Taq polymerase reaction buffer, respectively. Each PCR cycle contained 94°C, 50 s; 60°C, 1 min; 72°C, 1 min; and finally 72°C, 8 min. β-actin was used as a control. The amplified fragments were detected by agarose gel electrophoresis and visualized by ethidium bromide (EB) staining. The oligonucleotide primers used were: For VDAC, sense 5'- GGC TAC GGC TTT GGC TTA AT -3' and anti-sense 5'- CCC TCT TGT ACC CTG TCT TGA -3', yielding a deduced amplification product of 321 bps; while, for β-actin, sense 5'- TGC TAT CCC TGT ACG CCT CT -3' and anti-sense 5'- GGA GGA GCA ATG ATC TTG A -3' yielding a deduced amplification product of 601 bps.

2.9 Western Blot analysis for VDAC

Liver samples were homogenized in ice-cold lysis buffer. Homogenates were centrifuged at 12,000 g for 10 min and the supernatants were collected and the protein concentration was determined using Coomassie Brilliant Blue. The samples (40 µg per lane) were dissolved in sample buffer and separated by 12% SDS- polyacrylamide gel electrophoresis (PAGE) gels and electrophoretically transferred onto a polyvinylidene-difluoride (PVDF) membrane (Bio-Rad). The membrane was incubated with VDAC primary antibody (1:4000) and β-actin antibody (1:80000). The membrane was then exposed to the enhanced chemiluminescence (ECL) solution.

2.10 Statistical analysis

Differences among all groups were analyzed by one-way analysis of variance (ANOVA), followed by SNK-q-test . A P value <0.05 was accepted as statistically significance.

3. Results

3.1 Inhibition in the elevation of sALT and sAST level induced by CCl4

Marked elevation in both sALT and sAST activities was observed after injection of CCl$_4$, while 100 mg/kg, 200 mg/kg and 400 mg/kg LSE significantly blocked the sALT and sAST increase, especially the 200 mg/kg and 400 mg/kg LSE treatments, which maintained the sAST almost at normal level.(Fig. 1).
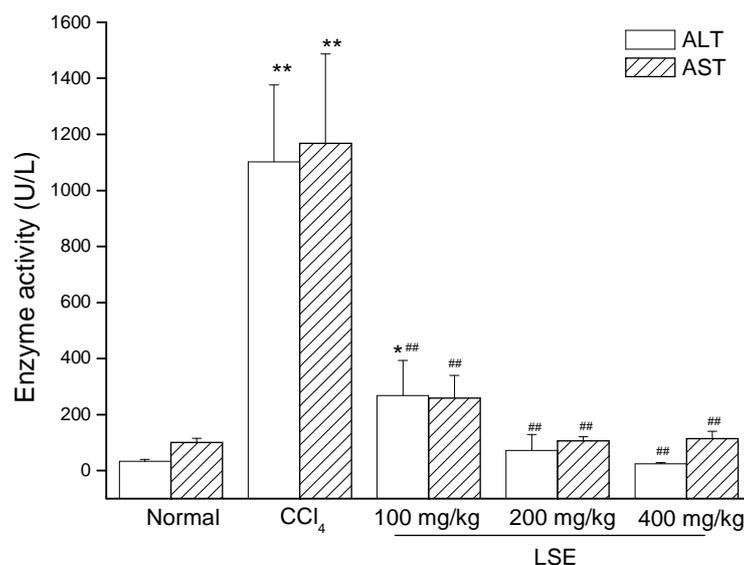
Figure 1. Effect of LSE on the elevation of serum AST and ALT activities in CCl₄ insulted mice.
Mice were divided into groups as follows: Normal, CCl₄, 100 mg/kg LSE, 200 mg/kg LSE and 400 mg/kg LSE. CCl₄ and different LSE groups were orally treated with saline or various concentrations of LSE for 5 d prior to the intraperitoneal injection with CCl₄. The blood samples were obtained 18 h later. Each value represents mean ± S.D. of 8 mice. *$P<0.05$, **$P<0.01$ vs. Normal, $^{\#\#}P<0.01$, vs. CCl₄ group.

3.2 Protection on the ultrastructure of liver insulted by CCl4

Compared with the normal group, obvious ultrastructure changes such as mitochondrial swelling and fragmentation of the cristae, lipid deposition and nuclear condensation were observed in CCl₄-insulted mice. However, the ultrastructure of hepatocytes and liver mitochondria of mice administered 100 mg/kg was improved to some extent, and those in the 200 mg/kg or 400 mg/kg LSE groups were almost similar to the normal cells (Fig. 2).

3.3. Effect of LSE on mitochondrial membrane potential dissipation

Under the present experimental condition, the mitochondrial membrane potential of normal mice was -192.1 ± 5.9 mV, which dropped to -162.8 ± 7.8 mV (15.3 %, $P < 0.01$) when mice were intraperitoneally injected with CCl₄ (Fig. 3). LSE preserved the mitochondrial membrane potential in a dose-related manner. 100 mg/kg LSE started to reverse the membrane potential compared to CCl₄ group, but was not statistically significant. At a dose of 200 or 400 mg/kg, the mitochondrial membrane potential was restored to the level observed for normal mice.
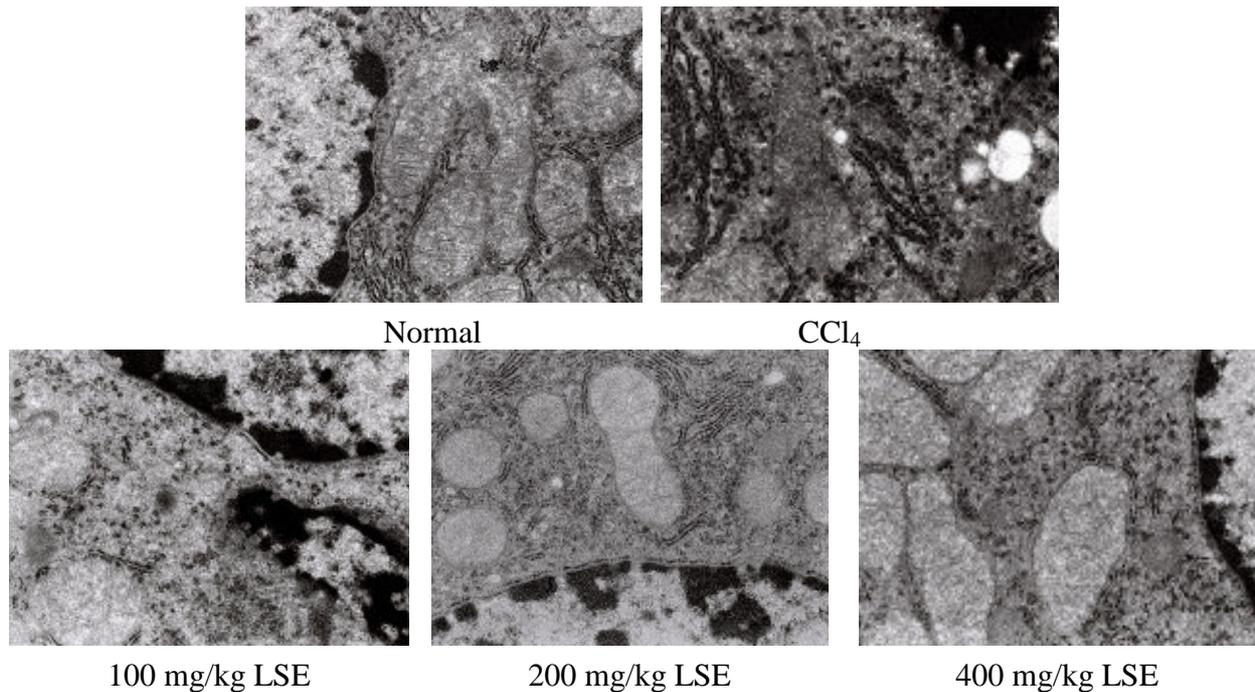
Figure 2. Effect of LSE on the ultrastructure of hepatocytes insulted by CCl4. Mice were divided into the same 5 groups as in Fig 1. Specimens were taken 18 h later and prepared for examination under an electron microscope (×12,000）. Compared with the normal group, mitochondrial swelling and fragmentation of the cristae, lipid deposition and nuclear condensation were observed in CCl4-insulted mice, which could be blocked to some extent by administration of LSE.

3.4 Effect of LSE on mitochondrial VDAC expression in CCl4-insulted mouse livers

Down-regulation on liver VDAC mRNA level in CCl4-stimulated mice

The effect of LSE on VDAC mRNA transcription was examined by RT-PCR. VDAC mRNA was detected in the normal group, which could be stimulated with CCl4 (Fig. 4A). Furthermore, LSE (100 mg/kg, 200 mg/kg and 400 mg/kg) significantly blocked the CCl4-stimulated VDAC mRNA elevation.

Down-regulation on liver VDAC protein level in CCl4-stimulated mice

The LSE-mediated down-regulation on VDAC protein expression was further corroborated by Western Blot (Fig. 4B). Normal animals showed a significant signal for VDAC, and mice receiving CCl4 showed a significantly stronger signal for VDAC. In contrast, in mice preadministered with LSE, a lower level of VDAC protein similar to that of normal mice was observed 18 h following CCl4 treatment, compared to mice with CCl4 alone.
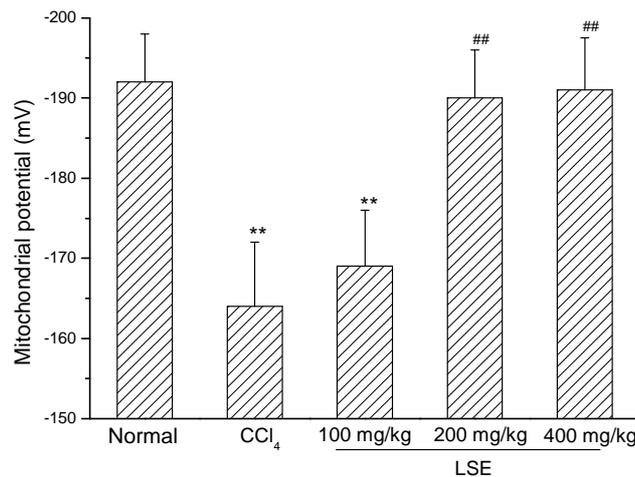
Figure 3. Prevention of LSE on mitochondrial membrane potential dissipation induced by CCl₄.
Mice were divided into 5 groups as Normal, CCl₄, 100 mg/kg LSE, 200 mg/kg LSE and 400 mg/kg
LSE. Livers from each group of mice were taken 18 h later. Liver mitochondria were isolated and the
mitochondrial membrane potential was determined using rhodamine 123. Each value represents mean
± S.D. of 8 mice. **P<0.01, vs. Normal, ##P<0.01, vs. CCl₄ group.

4. Discussion

The results of the present study show that 100 mg/kg, 200 mg/kg or 400 mg/kg LSE significantly
protect mice against CCl₄-induced hepatotoxicity, as demonstrated by its inhibition of the elevation of
sAST and sALT. Liver damage induced by CCl₄ is commonly used to screen drugs for
hepatoprotective activity [16,17]. CCl₄-induced acute liver injury may be initiated by the •CCl₃ radical,
which is formed by a metabolic enzyme (cytochrome P450) and could induce peroxidation of the
unsaturated fatty acids of cell membrane, and lead to membrane injury and leakage of enzymes such as
AST and ALT[10]. In fact, sAST and sALT are the most sensitive indicators of liver injury, with the
extent of hepatic damage assessed by the serum level of enzymes released from cytoplasm and
especially mitochondria [18].

(A)   (B)
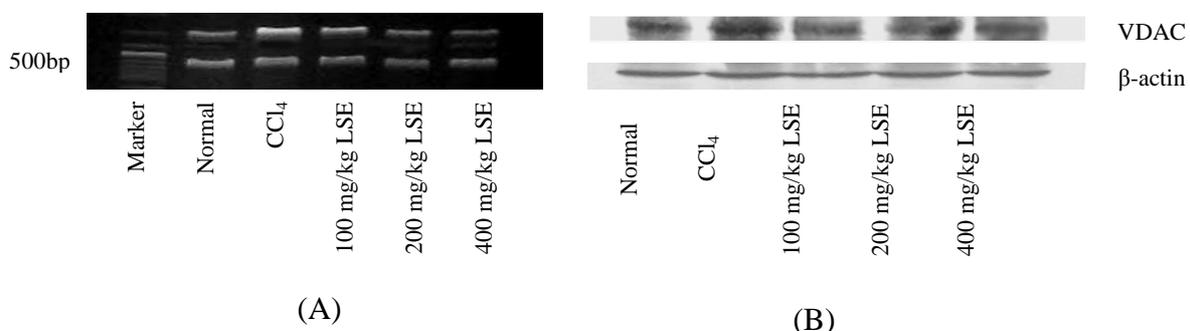
Figure 4. Effect of LSE on mitochondrial VDAC expression in CCl₄ -insulted mouse livers. Mice
were divided into Normal, CCl₄, 100, 200, 400 mg/kg LSE groups. Livers from various group mice
were taken 18 h later. (A) Inhibitory effect of LSE on the increase in VDAC mRNA level induced by
CCl₄, which was analyzed by RT-PCR. (B) Inhibitory effect of LSE on the increase in VDAC protein
level induced by CCl₄, which was analyzed by western blot. β-actin was used as a internal standard.

At the same time, the increase in sAST suggests a possible role for LSE on mitochondria, because 80% of sAST is released from mitochondria [18]. Based on our results, we speculate that LSE has a protective effect on both hepatocytes and their mitochondria, which was confirmed by ultrastructure examination. Protection of liver mitochondrial against hepatocytes injury induced by CCl_4 was demonstrated for LSE (200 mg/kg and 400 mg/kg).

Another sensitive marker of mitochondrial injury is the dissipation of the mitochondrial membrane potential [19]. In the present work, the protective effect of LSE for the liver mitochondrial membrane potential in CCl_4-intoxicated mice was evaluated. Treatment of mice with CCl_4 damaged liver mitochondria as demonstrated by the dissipation of mitochondrial membrane potential which is in accordance with our previous studies [5,6]. LSE from 200 mg/kg-400 mg/kg could significantly prevent the collapse of the mitochondrial membrane potential, confirming the protective effect of LSE against mitochondria deficiency.

Evidence was accumulated that there was change in the levels of expression of the mitochondrial VDAC, one of the most important proteins on the outer membrane regarding the process of apoptosis [8, 20-22]. VDAC levels increased significantly after CCl_4 administration and pretreatment of LSE could dose-dependently inhibit the elevation of both transcriptional and translational level of VDAC in the acute liver injury process, suggesting that the protective effect of LSE on liver mitochondrial in mice might be related to a down-regulation of the expression of mitochondrial VDAC which could be up-regulated by CCl_4.

In conclusion, the results of present study suggested that LSE has hepatoprotective activity and the mechanisms underlying its protective effects may be related to mitochondrial protection and especially the regulation of VDAC expression.

Acknowledgements

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