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Cantharidin-induced liver injuries in mice and the protective effect of vitamin C supplementation

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ABSTRACT

Cantharidin, a promising anti-cancer medication, is limitedly prescribed due to the risk of hepatic toxicity. Our previous study has shown that vitamin C (VC) acts as a potential hepatoprotective agent against chemical liver damage. Here we implemented further experiments to investigate the benefits of VC on cantharidin-induced liver injuries in mice. The findings showed that VC mitigated cantharidin-mediated hepatic impairments via reducing liver enlargement, as well as lowering elevated serum concentrations of glutamic-pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT), whereas the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), sodium-potassium ATPase (Na^+K^+-ATPase) in the liver was increased. In addition, the count of intrahepatic TNF-α positive cells was lowered. The mRNAs of TLR4 and NF-κB pro-inflammatory mediators were down-regulated. Moreover, the phosphorylation of IkB level was decreased in the hepatocytes, while the Mn-SOD (SOD2) expression was up-regulated. Overall, these observations demonstrate that vitamin C has pre-clinical benefits against cantharidin-induced liver injury, possibly through attenuating inflammatory response and oxidative stress.

1. Introduction

In the clinical practice, medication prescription may occur in drug-induced liver injury (DILI) because liver is susceptible to damage from medicine-derived toxins [1]. Statistically, DILI accounts for a relatively high proportion of organ failures, revealing the necessity for drug monitoring [2]. Drug metabolism-induced injury to hepatocytes causes deposition of bile acid and oxyradical/cytokines within the liver, thus further aggravating liver impairments [3]. Nowadays, there is a challenge in clinical practice to attenuate hepatotoxicity. Notably, partial herbal extracts can cause injury to the liver cells when prescribed within therapeutic ranges [4,5]. Cantharidin is a terpenoid compound extracted from blister beetle, which can medically be used as a medication for treating cancers, especially hepatocarcinoma [6]. Mechanistically, cantharidin-mediated therapeutic efficacy may be related to suppression of protein phosphatase 2A activity for inducing apoptosis in cancer cells [7,8]. As a poisonous agent, the adverse effects of cantharidin should be attenuated before it can be more safely used to manage cancers. Given the clinical practical need, effective strategy for weakening cantharidin-induced hepatotoxicity has been become urgently. Some studies have suggested that vitamin C, a vital cofactor that regulates enzymatic reactions, plays as a potent antioxidant against inflammation and oxidative stress through activating intracellular molecular pathways [9]. In addition, health benefits of vitamin C supplementation have provided positive advantages, such as prevention of metabolic disease or cancer [10,11]. More interestingly, our previous findings have indicated that VC has the potential capability of attenuating chemical or immunodeficient liver injury in mice [12,13]. In parallel, we speculate that VC would be beneficial for hepatoprotective effect against DILI. In the present study, the mouse was employed to establish a liver damage model induced by cantharidin, and therapeutic efficacy of VC would be investigated.

2. Materials and methods

2.1. Materials

Cantharidin (purity > 98%, determined with HPLC as indicated in Fig. 1) was purchased from Shanghai Jingke Chemical Technology Co., Ltd. VC (purity > 95%) was provided by Succhi Shiqi Pharmaceutical Co. Ltd. (Guangdong, China). Orderly, other required reagents/kits were listed as follows.

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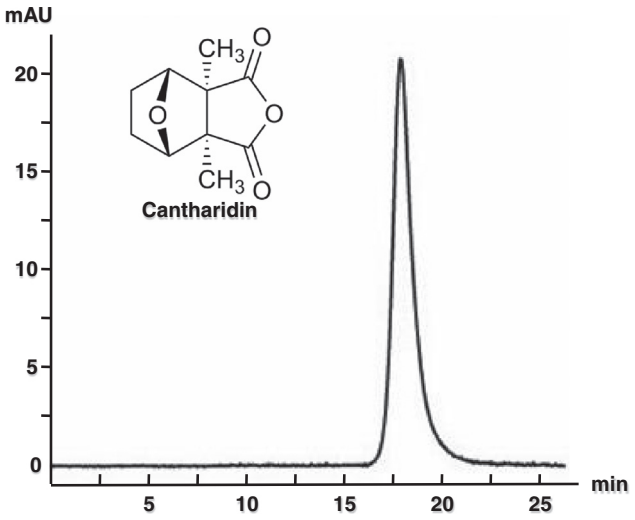

Fig. 1. Graph showed the molecular formula of cantharidin, in which the purity was determined using high-performance liquid chromatography (HPLC) before use.

2.2. Animals and VC delivery

All Kun Ming (KM) mice were male aged 6 weeks, weighted 25 ± 2 g, and obtained from the Experimental Animal Centre of Guangxi Medical University (Nanning, China). The mice were placed in the animal facility with a temperature of 25 ± 2 °C and a humidity of 60 ± 1% under a 12-h dark/light cycle. All mice were fed with standard chow and clean water.

For induction of DILI in animal, the mice were orally given 1.5 mg/kg body weight cantharidin (dissolved in hot-water via ultrasound for hydrotropy) for 14 days, in which designated dose was based on the conversion from clinical application. Meanwhile, the VC-treated groups (dissolved in 0.1% sodium metabisulfite; 100, 200 mg/kg; n = 10) were administered through intraperitoneal injection for 14 days. As a vehicle control, 10 health mice were given equal amounts of distilled water and solvent (0.1% sodium metabisulfite). All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee at the Guilin Medical University.

2.3. Sample collection

After 14 days, the mice were killed under anesthetic state conducted with 5% pentobarbital sodium (v/w). Serum samples were collected in heparinized tubes (30 U/mL). Isolated livers were dehematized with PBS and stored at −80 °C for further experiments.

2.4. Calculation of liver index

Mouse body weight was recorded and isolated liver was weighed. The liver enlargement condition was evaluated as follow:

$$\text{Liver index} = (\text{Liver weight}/\text{Body weight}) \times 100\%$$

2.5. Determination of transaminase enzymes

Serological test aimed to identify the concentrations of GPT, GOT according to manufactory protocols (Nanjing Jiancheng Bioengineering Institute, China), and representative results were indicated as U/L.

2.6. Determination of liver homogenate parameters

The activity of Na^+K^+-ATPase in liver homogenate was measured via a Minim-ATP enzyme test kit (Nanjing Jiancheng Bioengineering Institute, China). After the treatment, liver Na^+K^+-ATPase activity was assayed via calculating the number of inorganic phosphate with malachite green dyemethod using a spectrophotometer at 636 nm. And representative results indicated as U/mg protein. In addition, measurement of intrahepatic SOD and GSH-P_X levels were conducted as described previously [13].

2.7. Histology observation

5 μm live sections were subjected to routine staining with hematoxylin and eosin (H&E). Histopathological changes were screened and the images were captured (CX41, Olympus, Japan). In addition, cell necrotic occurred in the liver cells was assessed following previous reference [13].

2.8. Immunostaining procedures

Hepatic sections were pre-treated via different concentrations of ethanol and xylene (65%, 80%, 90%, 100%) for 5 min each time. After inactivating endogenous enzymes, antigen retrieval was performed in liver samples via heating. When blocking with freshly prepared 5% bovine serum albumin, the sections were incubated with primary rabbit-anti-mouse antibody (TNF-α, 1:500, Boster, Wuhan, China) before adding strept avidin–biotin–peroxidase complex (SABC, Boster, China). After rinsing with PBS, the samples were exposed to freshly prepared 3, 3′-diaminobenzidine for chromogenic reaction and then counterstained with hematoxylin, followed by the steps of dehydrating, permeabilizing, and mounting. The images were captured using a light microscope (Leica, German). In addition, data were averaged under 5 non-overlapped areas via counting the positive cells.

2.9. TLR4 and NF-κB mRNAs tests

Total RNAs were extracted from liver specimen using a Trizol kit (Life Technologies Corporation, USA) following manufacturer's protocols. RNA purity was determined by a spectrophotometer at absorbance of 260/280 nm. The step from RNA to cDNA transcription was completed by using commercial cDNA Synthesis kit (Life Technologies Corporation, USA). Subsequently, target primer sequences were provided as follows: β-actin sense primer: 5′ TGT GTC CGT CGT GGA TCT GA 3′, antisense primer: 5′ TTG CTG TTG AAG TCG CAG GAG 3′ (149 bp); TLR4 forward primer, 5′ CAT GGA TCA GAA ACT CAG CAA AGT C 3′, antisense primer: 5′ CAT GCC ATG CCT TGT CTT CA 3′ (179 bp); NF-κB forward primer, 5′ GAACGATAACCTTTGCAGGC 3′, and reverse primer, 5′ TTTCGATTCCGC TATGTGTG 3′ (130 bp). PCR reaction was conducted 30 cycles of denaturation at 92 °C for 30 s, annealing 45 °C for 30 s, and elongation at 72 °C for 2 min. The PCR product was loaded in sepharose gels and performed electrophoresis for 30 min. Finally, optical density of gel was calculated compared with the β-actin to yield a relative value.

2.10. Western blotting

Liver protein extracts were prepared with pre-chilled RIPA buffer. Collected lysates were processed with centrifuge at 10,000 × g for 15 min, and protein concentration was measured through a commercial kit (Bio-Rad, USA). Commensurable number of protein (20 μg) was separated with 10% SDS–PAGE and transferred to PVDF membrane (Bio-Rad, USA). After being blocked with 5% non-fat milk solution for 1 h, the membrane was incubated with primary antibodies against p-IkB (1:500; Boster, China) and SOD2 (1:500; Boster, China), respectively. Rinsing with PBS for 3 times, the membrane was exposed to secondary antibodies (1:2000; Boster, China) at

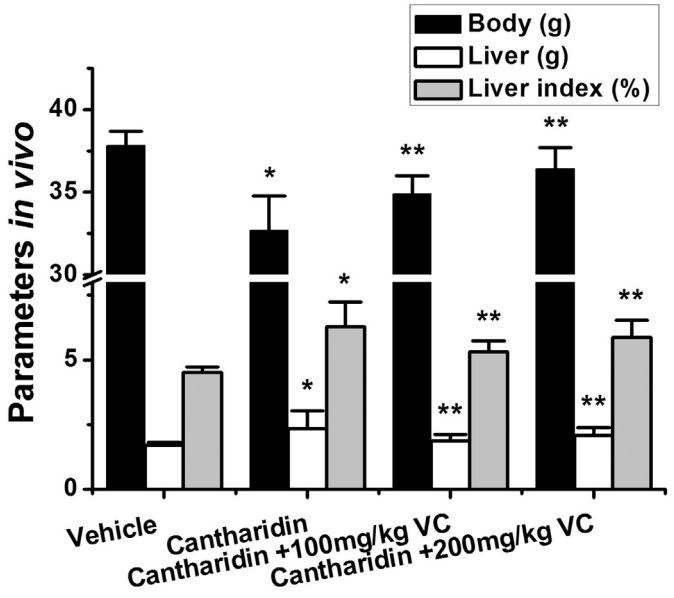

Fig. 2. *VC improved the damaged signs induced by cantharidin in mice.* Each experiment was performed repeatedly with two times, accompanied with five mice use in each group. Data are analyzed by one-way ANOVA followed by Bonferronni post tests, and outcomes indicate as the mean ± SD. Notes: vs. vehicle control, $^{*}P < 0.05$; vs. cantharidin-treated control, $^{**}P < 0.05$.

37 °C for 1 h and then conducted ECL detection (Beyotime, China). Final protein yield was expressed as a relative intensities compared to the control band.

2.11. Statistical analysis

The statistical results were performed via SPSS 13.0 software (SPSS Inc., USA). Differences between groups were evaluated using a one-way analysis of variance (ANOVA) with Bonferronni post tests. Results were expressed as the mean ± SD. *P* value less than 0.05 was considered as statistically significant.

3. Results

3.1. VC protected mice from cantharidin induced weight loss in mice

At the end of experiments, the body weight in cantharidin-treated mice was significantly lowered when compared to the mice in vehicle control ($P < 0.05$). Interestingly, the cantharidin-VC-treated mice showed inhibition of weight loss induced by the cantharidin ($P < 0.05$). Additionally, the mice treated with cantharidin resulted in elevated liver index associated with hepatomegaly. After co-administration of cantharidin and VC, the mice showed reduced liver index and hepatic swelling ($P < 0.05$) (Fig. 2).

3.2. VC improved the serological and homogenate parameters in cantharidin-treated mice

After 14 days, liver functional aminotransferases, such as GPT and GOT, were notably increased, while the liver homogenate levels of SOD, GSH-Px, and Na^+/K^+-ATPase were reduced when compared to the vehicle control ($P < 0.05$). Following co-treatment with cantharidin and VC, the mice showed normalization of hepatic enzymes and

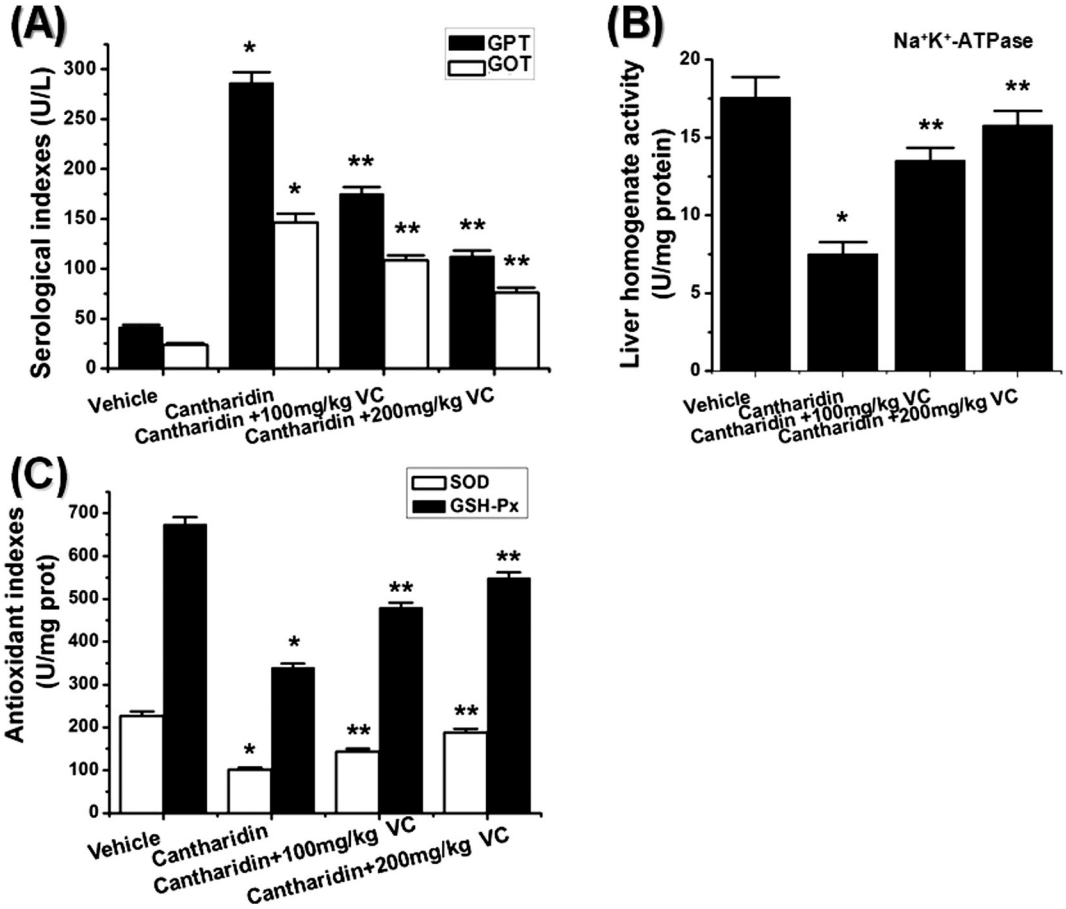

Fig. 3. (A) *VC corrected abnormal serum levels of GPT and GOT in cantharidin-damaged livers.* **(B–C)** *VC increased liver homogenate contents of Na^+/K^+-ATPase, SOD, GSH-Px in cantharidin-treated mice.* Each experiment was performed repeatedly with two times, accompanied with five mice use in each group. Data are analyzed by one-way ANOVA followed by Bonferronni post tests, and outcomes indicate as the mean ± SD. Notes: vs. vehicle control, $^{*}P < 0.05$; vs. *cantharidin*-treated control, $^{**}P < 0.05$.

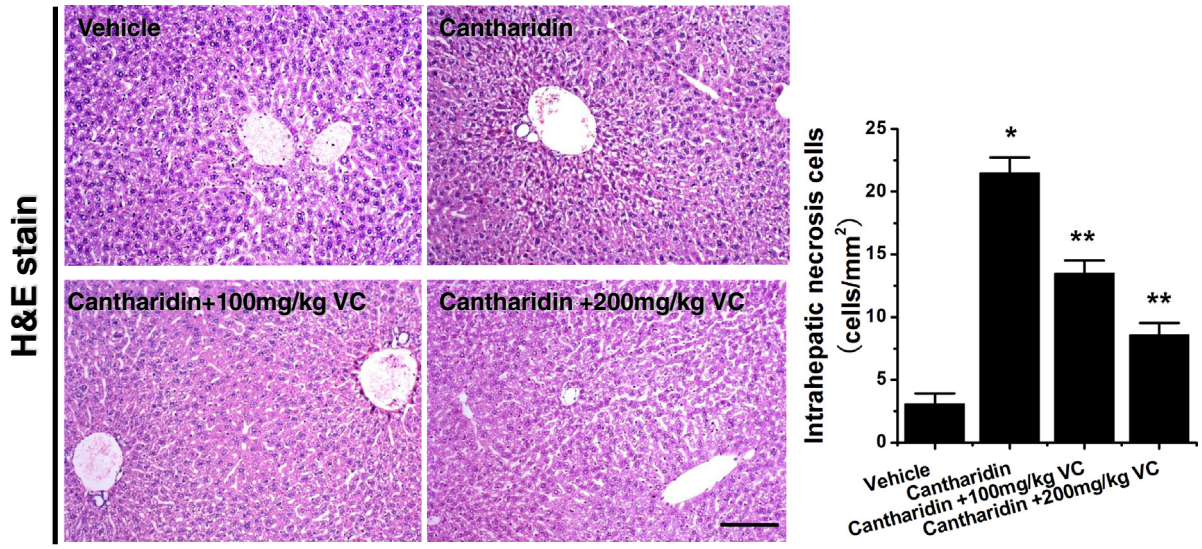

Fig. 4. *Morphological examination using routine H&E stain* (scale bar = 200 μm). Each experiment was performed repeatedly with two times, accompanied with five mice use in each group. Data are analyzed by one-way ANOVA followed by Bonferroni post tests, and outcomes indicate as the mean ± SD. Notes: vs. vehicle control, $^{*}P < 0.05$; vs. *cantharidin*-treated control, $^{**}P < 0.05$.

elevation of SOD, GSH-Px, and $Na^{+}K^{+}$-ATPase activities when compared to the cantharidin-damaged livers ($P < 0.05$) (Fig. 3).

3.3. Histopathological judgment

To morphologically characterize the changes in co-treated liver cells, routine stain was performed. The vehicle control mice had abundantly well-formed hepatocytes, which hepatic nuclei were rounded with visible nucleolus and scattered endothelial cells were distributed around the portal area. As a comparison, cantharidin-damaged liver showed pathologically impaired hepato-architectures, including deformed liver lobule and sinus hepaticus, reduced liver cells, deposited cytonecrosis, and inflammatory infiltration. In addition, the necrosis count was higher than that in vehicle control. Following the co-administration of VC, the cantharidin-damaged mice with liver impairments was reversed, marked by attenuated inflammation infiltration, improved cell structure, and reduced necrotic mass (Fig. 4).

3.4. VC reduced TNF-α immunoreactive cells in livers

As revealed in immunohistochemical staining, cantharidin-damaged livers showed elevated TNF-α immunoreactive cells, which the change was significant when compared to that in vehicle control ($P < 0.05$). Interestingly, the abnormal alterations were reversed in cantharidin-treated livers following with VC supplement ($P < 0.05$) (Fig. 5).

3.5. VC down-regulated mRNA expressions of TLR4 and NF-κB in cantharidin-treated livers

Further, the inflammation mediators were screened in the hepatocytes. As a consequence, cantharidin-damaged livers showed up-regulation of TLR4 and NF-κB mRNAs, which the levels were higher than those in vehicle livers ($P < 0.05$). After co-treatment with VC, the cantharidin-treated livers displayed lowered expressions of TLR4 and NF-κB mRNAs in a dose-dependent manner ($P < 0.05$) (Fig. 6A).

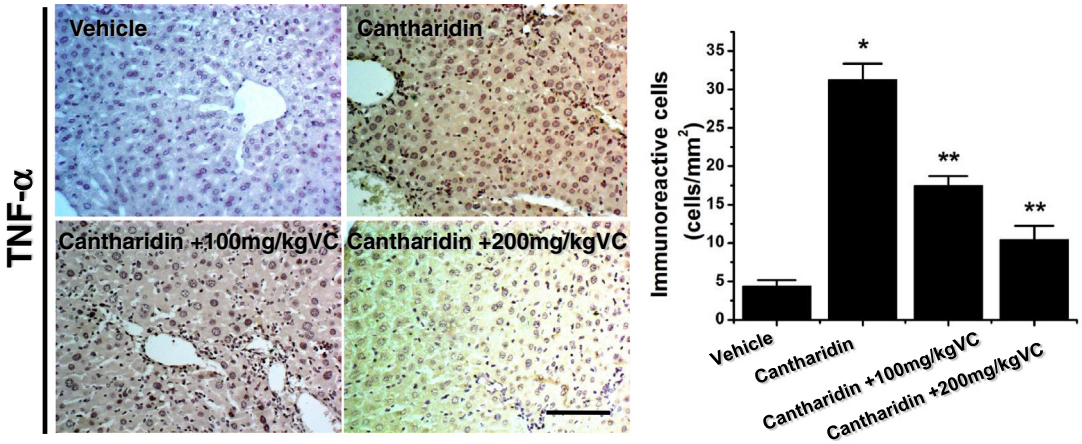

Fig. 5. *VC lowered TNF-α immunoreactive cell counts in the cantharidin-damaged livers* (immunohistochemistry, scale bar = 200 μm). Each experiment was performed repeatedly with two times, accompanied with five mice use in each group. Data are analyzed by one-way ANOVA followed by Bonferroni post tests, and outcomes indicate as the mean ± SE. Notes: vs. vehicle control, $^{*}P < 0.05$; vs. *cantharidin*-treated control, $^{**}P < 0.05$.

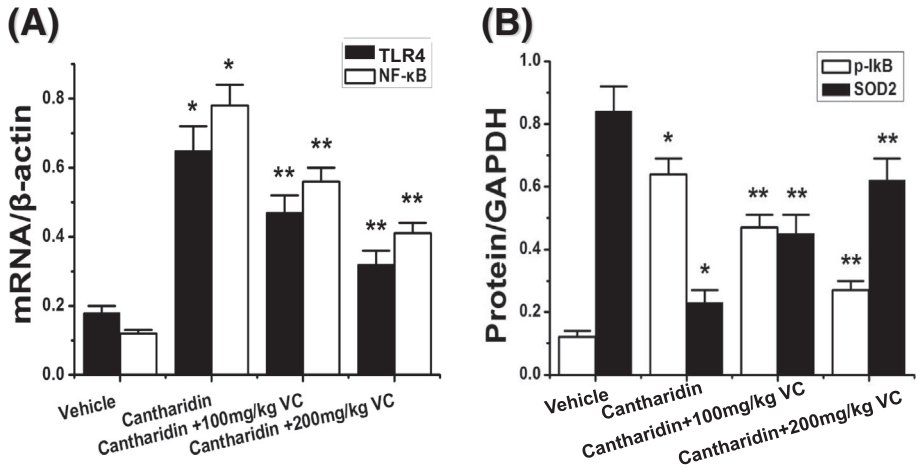

Fig. 6. (A) *VC reduced expressions of TLR4 and NF-κB mRNAs in cantharidin-damaged livers* (PT-PCR test). **(B)** *VC down-regulated p-IkB expression and elevated SOD2 levels in cantharidin-treated livers* (Western blot assay). Each experiment was performed repeatedly with two times, accompanied with five mice use in each group. Data are analyzed by one-way ANOVA followed by Bonferroni post tests, and outcomes indicate as the mean ± SD. Notes: vs. vehicle control, $^{*}P < 0.05$; vs. *cantharidin*-treated control, $^{**}P < 0.05$.

3.6. VC lowered p-IkB expression and up-regulated SOD2 levels in cantharidin-treated livers

As shown in Fig. 6B, cantharidin-damaged livers led to elevation of p-IkB and in turn down-regulation of SOD2 expression ($P < 0.05$). Following the VC supplementation, the abnormal expressions of p-IkB, SOD2 were inhibited, showing reduction of p-IkB and elevation of SOD2 levels in the livers ($P < 0.05$).

4. Discussion

In summary, the current findings demonstrate that VC exerts the potential hepatoprotective effects against liver injury induced by cantharidin, contributing to the future medical uses of VC. Thus, the details involved molecular mechanism needs to be discussed.

Existing clinical medications, when ingested overdoses or even within therapeutic ranges, may damage the liver [14]. Cantharidin has the pronounced adverse effects when used medically as an antineoplastic, because of the potential hepatotoxicity [15]. In this study, cantharidin-treated mice resulted in body weight loss and liver expansion, implying that the mice might have been sick and stopped drinking during the course of cantharidin-induced hepatic impairments. Promisingly, VC co-treated mice showed alleviated hepatomegaly, indicating that the benefits of VC supplement were due to improving morbid physical signs, such as reducing liver tissue edema and vascular congestion.

Clinically, determination of serum concentrations of transaminases is a vital method in diagnosing and identifying various diseases, such as liver injury [16,17]. In mammal, intracellular Na^+-K^+-ATPase can be physiologically responsible for animal cell's energy consumption. In addition, reduction of Na^+-K^+-ATPase level in the liver is related to hepatic injury/trauma that causes cellular toxicity [18,19]. Evidence has suggested that intrahepatic antioxidant units, including SOD and GSH-Px, serve as the potent ROS-scavenger to protect the cells from oxidative damage [13]. As a consequence, cantharidin-damaged liver showed abnormal elevation of hepato-functional enzymes and reduced intrahepatic Na^+-K^+-ATPase, SOD, and GSH-Px concentrations, indicating that cantharidin-induced hepatic impairment resulted in metabolic disturbance associated with intracellular energy barrier. More interestingly, VC co-treatment contributed to inhibition of cantharidin-induced hepatocellular dysfunctions, which were possibly related to maintenance of liver metabolic homeostasis and suppression of oxidative stress.

The state of inflammation is a response of organs to damage or harmful stimuli, which inflammatory mediators participate in tissue injury and cell necrosis [20,21]. Tumor necrosis factor (TNF-α), a known inducer of NF-κB activity, results in induction of inflammation response [22]. Notably, over-expression of TNF-α in the liver can be linked to hepatic injuries. Cytologically, protein complex NF-κB plays the important effect in cytokine expression and cell survival. Incorrect expression of NF-κB relates to the development of cancer, inflammation, and immunological diseases [23,24]. Reports have indicated that stimulation of TLR4, a specific pattern recognition molecule, induces NF-κB activation for initiating deleterious inflammatory cascading events [25,26]. When IκB subunit is degraded from the heterodimer, and then NF-κB complex enters the nucleus and triggers specific gene activities binding site for NF-κB. The targeted genes activated by NF-κB can cause serial physiological responses, such as inflammatory or immune response [27,28]. Thus, inactivation of NF-κB/TLR4 in the liver may be contributive to amelioration of hepato-functions. The present findings showed that cantharidin-damaged liver led to abnormal up-regulation of intrahepatic TNF-α, NF-κB, p-IκB, and TLR4. It reflected post-signs of liver damage which was consistent with increased necrotic hepatocytes and inflammation infiltration as shown in HE stain. After co-treatment with VC, the liver impairments associated with abnormal expressions of regulatory gene/protein were suppressed.

Collectively, VC supplement may attenuate cantharidin-induced hepatotoxicity and the molecular mechanisms are involved in suppression of intrahepatic oxidative stress and TLR4/NF-κB pathway associated with inflammation, thereby ameliorating metabolic functions in liver cells. In the future, more studies merit further implementation prior to clinical application of VC against drug-induced liver injury.

Declaration of interest

The authors declare they have no competing financial interests.

Acknowledgments

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