Liver Injury Induced by Cantharidin Through Endoplasmic Reticulum Stress, Autophagy, and Apoptosis in Rat

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ABSTRACT: OBJECTIVE To explore the toxicological mechanism of drug-induced liver injury(DILI) in rats induced by cantharidin(CTD). **METHODS** SD rats were exposed to different doses of CTD(0.061 4, 0.092 1, 0.184 1 mg·kg^{-1}) by oral gavage for 28 d. Liver index and serum liver function indictors were detected. HE staining was used to evaluate the pathological changes of liver. Then the proteins in endoplasmic reticulum stress(ERS), autophagy, and apoptosis-pathway were detected by Western blotting. **RESULTS** The liver index was increased in CTD groups. The ALT, AST, LDH, ALP and T-Bil were increased by CTD with a dose-dependent manner. Disrupted hepatic architecture and dilatation of central vein were observed after CTD intervention. The protein expression levels of GRP78, CHOP, ATF4, Beclin-1, LC3, Caspase-3, Caspase-8, and Bax/Bcl-2 were increased after CTD intervention. Molecular docking results revealed that GRP78, ATF4, and Beclin-1 could directly interconnect with CTD. **CONCLUSION** CTD can activate ERS, autophagy and synergistically inducing downstream apoptosis in rat, providing a novel insight into the mechanism of CTD-induced DILI.
KEYWORDS: cantharidin; drug-induced liver injury; endoplasmic reticulum stress; autophagy; apoptosis

内质网应激、自噬与凋亡在斑蝥素致大鼠肝毒性中的作用

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摘要：目的 探讨斑蝥素 (cantharidin，CTD) 致大鼠药物性肝损伤 (drug-induced liver injury，DILI) 的毒理学机制。**方法** 采用不同剂量 CTD(0.061 4，0.092 1，0.184 1 mg·kg^{-1}) 连续灌胃 SD 大鼠 28 d，检测肝脏指数和血清肝功能指标，HE 染色评估肝脏病理变化。进一步采用免疫印迹法检测内质网应激 (endoplasmic reticulum stress，ERS)、自噬和细胞凋亡通路蛋白。**结果** CTD 干预后肝脏指数显著升高，生化指标 ALT、AST、LDH、ALP 和 T-Bil 显著升高，且呈剂量依赖性，肝脏组织出现结构破坏和中央静脉扩张等病理变化；GRP78、CHOP、ATF4、Beclin-1、LC3、Caspase-3、Caspase-8 和 Bax/Bcl-2 的蛋白表达水平显著升高。分子对接结果显示，GRP78、ATF4 和 Beclin-1 与 CTD 对接结果良好。**结论** CTD 可激活大鼠 ERS，进一步激活自噬，诱导下游凋亡，研究结果可为 CTD 诱导的 DILI 提供新的科学依据。
关键词：斑蝥素；药物性肝损伤；内质网应激；自噬；凋亡
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Natural herbs gaining popularity in world in the latest decades due to its safety and effectivity[1-2]. However, after widespread clinical application of natural herbs, the safe and adverse reactions have received widespread attention, especially in drug-induced liver injury(DILI)[3-4]. Data show that herbal-induced liver injury accounts for 20% of cases of hepatotoxicity in the United States, which has been drawn growing attention worldwide[5].

Mylabris, also called "*Spanish fly*", is a widely distributed blister beetle of the Meloidae family[6-7], which has been used in tumor treatment as a natural

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medicine in China for thousands years. Modern pharmacology study revealed that cantharidin(CTD) is the major component with strong anti-tumor effect on liver, gastric, and lung cancer[8-10], which has been developed into modern preparations as "Aidi injection and Compound Banmao capsule"[11-12]. However, the clinical use of Mylabris and its preparations could cause DILI, which limited the application in tumor treatment[13]. A clinical report showed that a rectal cancer patient has suffered DILI with liver function damaged after oral administration of Compound Banmao capsule[14]. Moreover, animal experiments also showed that CTD could induce DILI with liver necrosis, inflammatory infiltration and hemorrhage[15]. Recent studies have showed that oxidative stress, inflammation, metabolism disorders were the toxic mechanism in CTD-induced DILI[15-18].

Moreover, CTD-induced DILI in rat was related to lipids disorders, which demonstrated that lipids disorders may play a role in DILI of CTD[19]. Deeply, endoplasmic reticulum stress(ERS) is considered to be a key regulation upstream process in lipid metabolism[20-21]. And then ERS could induce inflammatory, and apoptosis to cause DILI[22]. Meaningfully, study showed that various doses of CTD(≥0.392 μg·mL^{-1}) could induce DILI via activating ERS in LO2 cells for 24, 48, 72 h[23]. On the contrary, the previous study showed that CTD(25, 50 μmol·L^{-1}) could induce LO2 cell toxicity via inhibiting ERS, activating autophagy and apoptosis in 6 h and 12 h[24]. Maybe the ERS pathway could be affected with various action by the intervention dose and time by CTD in the LO2 cell, which needed to be fully elucidated *in vivo*.

In this study, the role of ERS in CTD-induced DILI in rat was explored, which was combined with molecular docking, providing a basis in regulating protein activation by ERS with autophagy and apoptosis *in vivo*.

1 Materials and methods

1.1 Reagents and instruments

CTD(Sigma, USA, lot: C7632; purity: 99%); RIPA lysis buffer(Solarbio, China); BCA kit(Jian Cheng Bioengineering Institute, China, lot: A045-4); Primary antibodies: glucose-regulated protein-78(GRP78)(lot: 3183), C/EBP-homologous protein (CHOP)(lot: 2895), activating transcription factor 4(ATF4)(lot: 11815), Beclin-1(lot: 3495), microtubule-associated protein 1 light chain 3(LC3)(lot: 12741), Caspase-3(lot: 14220), Caspase-8(lot: 4790), goat anti-rabbit IgG secondary antibodies(lot: 7074) all purchased from CST, USA; B-cell lymphoma 2(Bcl-2)(Abbkine, China, lot: ABM0010); Bax antibodies(lot: ET1603-34), β-actin(lot: A01010) all purchased from HuaAn, China; ECL kit(Shanghai 7Sea Pharmatech, China, lot: E003-050). AU5800 Automatic biochemical analyzer (Beckman, USA); BX43 Flight microscope(Olympus, Japan); Sorvall Legend Micro 21R(Thermo Fisher, r=8.8 cm).

1.2 Animal grouping and intervention

32 SD rats (180±20)g with half female and half male were purchased from Changsha TianQin Laboratory Animal Co., Ltd.[Production license number: SCXK(湘)2014-0011]. After adaptive feeding, 32 rats were randomly divided into the control and the 3 doses of CTD groups. The rats in CTD groups were received different doses of CTD(CTDL,0.061 4 mg·kg^{-1};CTDM,0.092 1 mg·kg^{-1}; CTDH, 0.184 1 mg·kg^{-1}) by oral gavage daily for 28 d, respectively. The rat in control group was received a same volume of CTD with 0.5% CMC-Na; rats were housed on a 12 h light/dark cycle under conditions of controlled temperature(24 ℃) and humidity with adlibitum access to standard laboratory chow food and water.

At the end of the experiment, all rats were anesthetized with urethan and sacrificed after fasting for 8 h. The blood from the abdominal aorta was allowed to stand at room temperature for 30 min, then was centrifuged at 4 ℃ for 15 min at 3 000 r·min^{-1} to obtain serum and storage at −80 ℃. The liver tissues were weighed and cut into pieces, and a portion of the liver tissue was fixed in 4% paraformaldehyde. The rest of the liver tissues were frozen in liquid nitrogen at −80 ℃. All animal experiments were approved by the Animal Ethics Committee of Zunyi Medical University(Zunyi, China, ZMUER2017-2-235). The

experimental steps complied with the Chinese National Guidelines for the Ethical Review of Laboratory Animal Welfare.

1.3 Liver index

The body weights of rats were recorded and the isolated livers were weighed. A liver index was evaluated as follows:

$$\text{Liver index} = \frac{\text{liver weight}}{\text{body weight}} \times 100\%$$

1.4 Serum biochemical detection

The alanine transaminase(ALT), aspartate aminotransferase(AST), total bilirubin(T-Bil), lactate dehydrogenase(LDH), and alkaline phosphatase(ALP) of rat serum samples were measured to evaluate liver function after CTD intervention by an automatic biochemical analyzer.

1.5 Liver histological examination

The liver tissue from each rat was fixed in 4% polymethyl aldehyde for 24 h. Paraffin-embedded liver tissues were stained with Hematoxylin/Eosin(HE) according to standard procedures. The histological images were captured using a light microscope.

1.6 Western blotting analysis

Total proteins were extracted from liver tissues using RIPA lysis buffer and quantified by BCA kit. The proteins were resolved using SDS-PAGE and then transferred to PVDF membranes. Membranes were blocked with 5% nonfat milk for 1 h, followed by incubation at 4 ℃ overnight with the following primary antibodies: GRP78(1∶1 000), CHOP(1∶1 000), ATF4(1∶1 000), Beclin-1(1∶1 000), LC3(1∶1 000), Caspase-3(1∶1 000), Caspase-8 (1∶1 000), Bcl-2(1∶1 000), and Bax(1∶1 000) antibodies. Then membranes were seeded with goat anti-rabbit IgG secondary antibodies(1∶2 000) at room temperature for 1 h and visualized with the ECL kit. The relative protein expression was normalized to β-actin(1∶5 000). Image lab software was utilized to analyze the bands.

1.7 Molecular docking

To verify the activated proteins in ERS and autophagy, molecular docking was performed to verify the effects between CTD and the targets. The structure of CTD was collected from Pubchem database(https://pubchem.ncbi.nlm.nih.gov/). The crystal structure of GRP78, ATF4, and beclin-1 proteins was obtained from the Protein Data Bank database[25](http://www.rcsb.org/). Finally, molecular docking was performed with AutoDock1.5.6 software and the docked models were analyzed using PyMol2.5. The lower the binding energy, the better the docking was bound. The binding energy< −5.0 kcal·mol^{-1}(1 cal=4.186 J) indicated that CTD binds well to the target.

1.8 Statistical analysis

Data was presented as the $\bar{x} \pm s$. All statistical analyses were performed using GraphPad Prism 9.0 software. For comparisons, one-way analysis of variance(ANOVA) was used. P<0.05 indicates a statistically significant difference.

2 Results

2.1 CTD-induced liver dysfunction abnormal in rats

The body weight in the CTDM group was decreased compared to the control group(P<0.05) (Fig. 1). The liver index was increased in the CTDL, CTDM, and CTDH groups compared to the control group(P<0.01), suggesting that hepatic edema may be caused in rats after CTD intervention. In addition, compared with control group, serum biochemical indicators including ALT, AST, LDH, and T-Bil were significantly increased in the CTDL, CTDM, and CTDH groups(P<0.05 or P<0.01). A significant increase of liver function levels is a clinical indicator of DILI[26], indicating that CTD could induce liver dysfunction and damage in a dose-dependent manner in rats.

2.2 Histopathological analyses

To morphologically characterize the liver of rat, HE staining was used. The results of liver sections showed that well-formed hepatocyte(HC), and the hepatic sinusoids(HS) were clearly visible in the control group(Fig. 2A). Notably, disrupted hepatic architecture was found in CTDL, CTDM, and CTDH groups, including various sizes of HC nuclei and slight changes in HC vacuoles(Fig. 2B, C). Moreover,

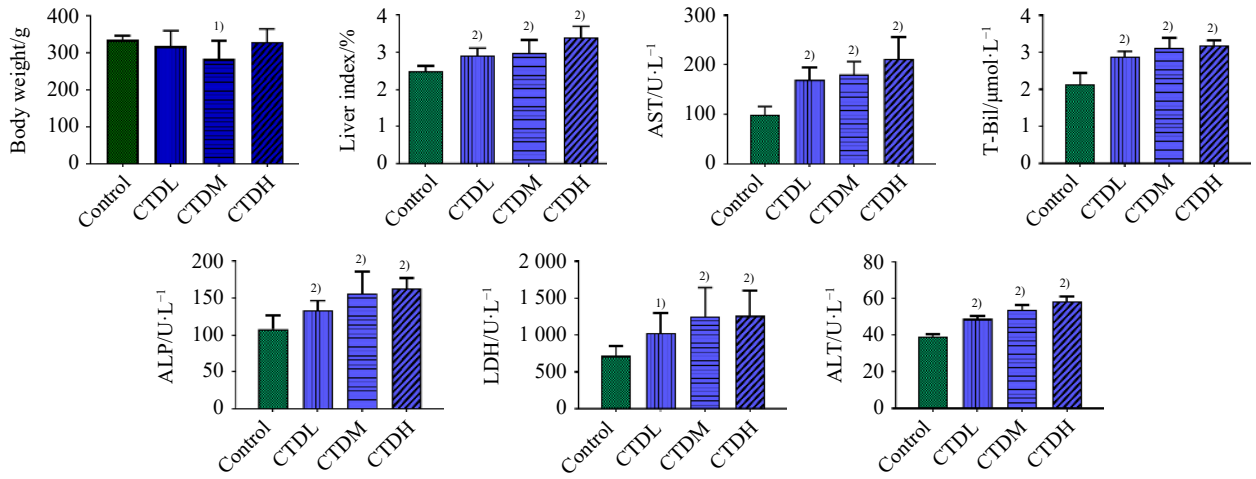

Fig. 1 Changes of body weight, liver index and liver function indicators in rats after cantharidin intervention($\bar{x} \pm s$, n=6−8)
Compared with the control group, [1)]P<0.05, [2)]P<0.01.

图 1 斑蝥素干预后大鼠体质量、肝脏指数、肝功能指标变化 ($\bar{x} \pm s$，n=6~8)
与对照组相比，[1)]P<0.05，[2)]P<0.01。

dilatation of central vein(CV) and HS, partial cell edema, and disordered arrangement of HCs were observed in CTDM and CTDH groups(Fig. 2D). Above results suggesting that CTD could induce liver injury in rat.

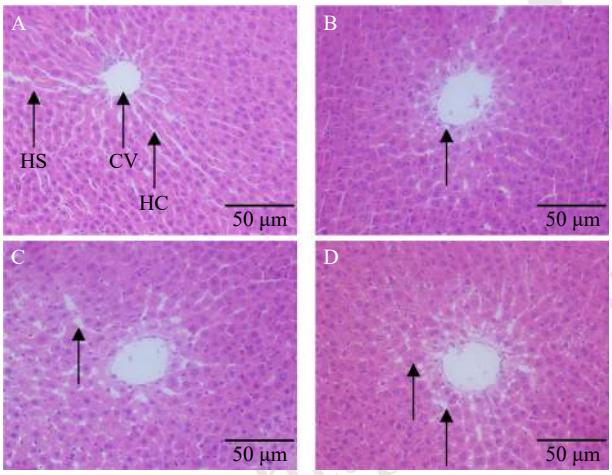

Fig. 2 HE straining of liver sections in rats after cantharidin intervention(arrow points to the location of pathological change)(400 ×)
A−control group; B−CTDL group; C−CTDM group; D−CTDH group; HC−hepatocyte cord; HS−hepatic sinusoids; CV−central vein.

图 2 斑蝥素干预后大鼠肝脏 HE 染色切片 (箭头指向病变位置)(400 ×)
A−对照组；B−CTDL 组；C−CTDM 组；D−CTDH 组；HC−肝细胞索；HS−肝血窦；CV−中央静脉。

2.3 Determination of ERS-, apoptosis-, autophagy-related proteins

In ERS pathway, compared with control group, the protein expressions of GRP78, ATF4, and CHOP were significantly increased in CTDL, CTDM, and CTDH groups with a dose-dependent manner(P<0.01), suggesting that CTD could induce DILI in rats through activating liver "ATF4-CHOP" ERS pathway(Fig. 3). In autophagy pathway, compared with control group, the protein expressions of LC3-Ⅱ/LC3-I and Beclin-1 were significantly increased in a dose-dependent manner in CTD groups(P<0.01)(Fig. 4). In apoptosis pathway, compared with control group, the protein expression of the Bax/Bcl-2 was increased in CTDL(P<0.05), CTDM, and CTDH groups(P<0.01), Caspase-3 were significantly increased in CTDL, CTDM, and CTDH groups(P<0.01) in a dose-dependent manner. And the protein expression of Caspase-8 was increased in CTDM(P<0.05) and CTDH groups(P<0.01), suggesting that rat liver apoptosis was activated after CTD exposure(Fig. 5). Above results showed that ERS, apoptosis, and autophagy in liver could be activated with a dose-dependent manner in CTD-induced DILI rat.

2.4 Molecular docking to observe the binding site

To further evaluate the affinity of CTD and functional proteins, GRP78, ATF4, and Beclin-1 were screened with greater binding energy using molecular docking. Of these, the binding energy of Beclin-1 was −6.018 kcal·mol^{-1}, which is lower than −5.0 kcal·mol^{-1} (Fig. 6A), indicating highly stable binding with CTD[27].

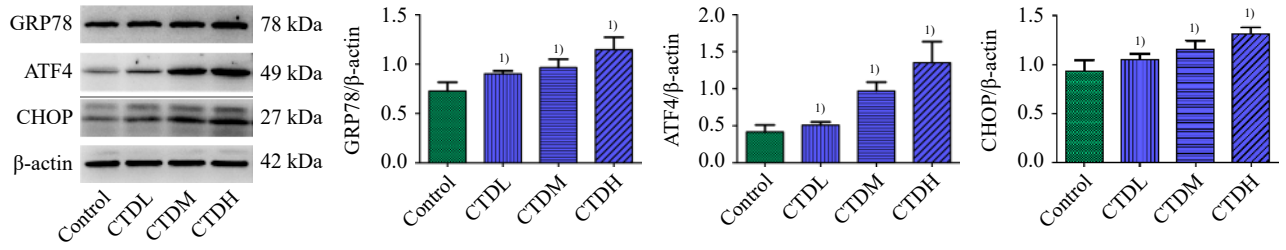

Fig. 3 Protein expression level of endoplasmic reticulum stress in rats after cantharidin intervention($\bar{x} \pm s$, n=6)

Compared with the control group, [1)]P<0.01.

图 3 斑蝥素干预后小鼠内质网应激蛋白表达变化 ($\bar{x} \pm s$，n=6)

与对照组相比，[1)]P<0.01。

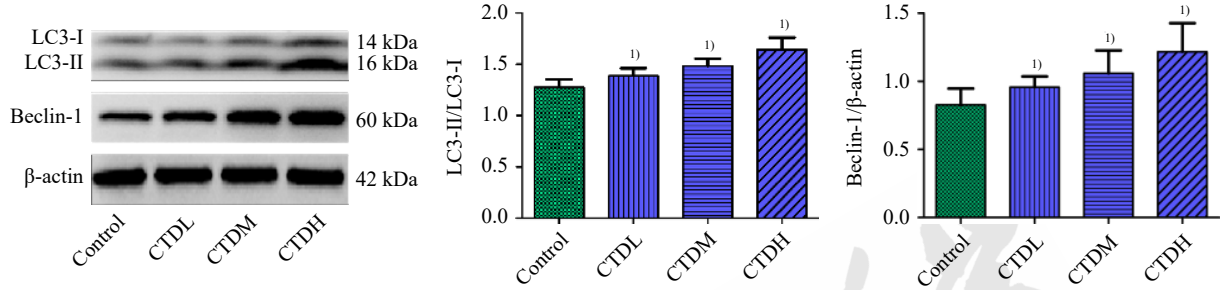

Fig. 4 Autophagy protein expression level in rats after cantharidin intervention($\bar{x} \pm s$, n=6)

Compared with the control group, [1)]P<0.01.

图 4 斑蝥素干预后小鼠自噬蛋白表达变化 ($\bar{x} \pm s$，n=6)

与对照组相比，[1)]P<0.01。

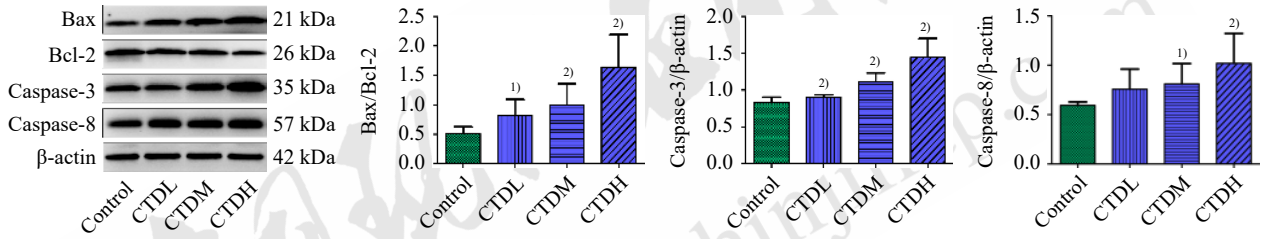

Fig. 5 Apoptosis protein expression level in rats after cantharidin intervention($\bar{x} \pm s$, n=6)

Compared with the control group, [1)]P<0.05, [2)]P<0.01.

图 5 斑蝥素干预后小鼠凋亡蛋白表达变化 ($\bar{x} \pm s$，n=6)

与对照组相比，[1)]P< .05，[2)]P<0.01。

The binding energy of ATF4 and GRP78 was −4.650 kcal·mol^{-1} and −4.390 kcal·mol^{-1}, respectively, which is lower than −4.250 kcal·mol^{-1}, suggesting that ATF4 and GRP78 has good bonding stability with CTD. Moreover, the docking pose showed that visible hydrogen bonds and strong electrostatic interactions were observed in CTD and Beclin-1(Fig. 6B), ATF4(Fig. 6C), and GRP78 (Fig. 6D), indicating highly stable binding.

3 Discussion

CTD is a classic anticancer component due to inhibition of protein phosphatase 2A, inducing inflammatory, and apoptosis[28-30]. The DILI of CTD has been gradually emphasized in recent years globally, which affect its clinical application[31]. Modern pharmacology studies found that vitamin C and Astragalus polysaccharides could partly attenuated CTD-induced DILI by inhibiting inflammatory response and regulating glycerophospholipid metabolism[17-18]. However, to date, more effective strategies for the prevention and treatment of CTD are still lacked due to the unclear mechanisms. In this study, systematic exploration of its effects of ERS, autophagy, and apoptosis pathway in DILI after the exposure to CTD *in vivo*, providing a basis for multiply hepatotoxicity mechanisms of CTD-induced DILI.

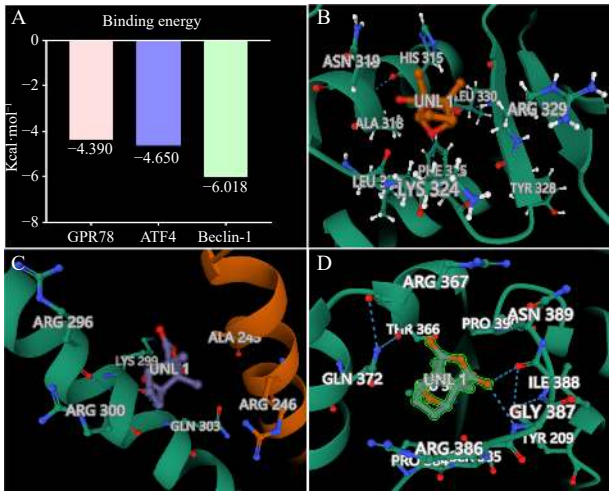

Fig. 6 Molecular docking(A) binding energy of molecular docking and best docking pose in Beclin-1(B), ATF4(C), and GRP78(D) with cantharidin

图 6 分子对接结合能(A)及斑蝥素与 Beclin-1(B), ATF4(C), GRP78(D)的对接形态

3.1 Toxicology evaluation of CTD

Different doses of CTD were utilized to evaluate the toxicity-effect relationship for the liver of rat. On the one hand, CTD could damage liver function with increased liver index including AST, ALT, LDH, T-Bil, and ALP levels in the CTDL, CTDM, CTDH groups with a dose-dependent manner in rats, which were generally recognized as hepatotoxicity markers, exhibiting an increasing activity when hepatocytes are necrotic[32-34]. On the other hand, disrupted hepatic architecture, dilatation of CV and HS, partial cell edema, and disordered arrangement of HCs were observed in the damaged liver after CTD intervention. Meaningfully, above results were similar to those of previous studies[19], suggesting that this CTD induced-DILI animal models were successfully constructed, which could be used for further mechanism researches.

3.2 ERS contribute to DILI of CTD

ERS is a result of increased unfolded proteins under diverse stimulating conditions especially in drug-toxicity[35]. Unfolded protein response(UPR) is mediated by an ER chaperone GRP78, so GRP78 detection is considered as a trigger of ERS. And ERS mainly includes three pathways of inositol-requiring enzyme 1, PKR-like ER kinase(PERK), and activating transcription factor 6(ATF6)[36]. Accumulating studies indicate that toxicity induction of liver is accompanied by activated ERS[37-40]. Furtherly, some studies reveal that "PERK-ATF4-CHOP" dependent ERS pathway is the major mechanism of DILI process of Saikosaponin and Pyrrolizidine alkaloids[41-43]. In this study, CTD increased the GRP78 protein expression, and then activated the "ATF4-CHOP" signaling pathway in the CTDL, CTDM, and CTDH groups in a dose-dependent manner, indicating that ERS was activated by CTD. Moreover, molecular docking results revealed that CTD could directly interacted with GRP78 and ATF4 with great binding energy, suggesting that CTD leading to DILI could be partially attributed to "ATF4-CHOP" ERS pathway.

3.3 Autophagy contribute to DILI of CTD

Autophagy is a catabolic machinery aimed at recycling cellular components and damaged organelles in response to toxic stress of drugs[44]. Currently, the pro-apoptosis role of autophagy in hepatotoxicity has been widely documented[45-47]. Activating autophagy has aroused concern in Acetaminophen, Isoniazid, and Triptolide-induced DILI[48-50]. During autophagy, LC3-I is modified and processed by ubiquitin-like system to generate LC3-II that is localized to autophagosomes, which is identified as a molecular marker of autophagy[51-52]. And Beclin-1 has a central role in autophagy which involved in autophagosome formation[53]. In this study, CTD increased the protein expression levels of LC3- II/LC3-I and Beclin-1 in the CTDL, CTDM, and CTDH groups in a dose-dependent manner, which might contribute to autophagosome formation and autophagy activating. And molecular docking result showed that Beclin-1 could be directly interacted with CTD, suggesting that CTD could induce autophagy in DILI rats, which furtherly supporting this role of activating autophagy.

3.4 Apoptosis in rat liver

Apoptosis is the described form of programmed cell death, which leads to the rapid demolition of cellular structures and organelles[54]. Apoptosis-induced cell death is the mainly mechanism in DILI, including *Tripterygium wilfordii*

multiglycoside, Toosendanin, and ochratoxin A-induced hepatotoxicity[49, 55-56]. As the important executioner caspase, Caspase-3 may reorganize the cytoskeleton and disintegrates cells into apoptotic bodies[57]. And TNF-α can lead to the recruitment and activation of Caspase-8 by extrinsic pathway, then cleaves pro-Caspase-3 to induce apoptosis. Caspase-8 also cleaves Bcl-2, which migrates to the mitochondria and forms Bax pores on its surface, releasing cytochrome C and inducing apoptosis[58]. In this study, CTD increased the protein expression levels of Caspase-3, and Bax/Bcl-2 in the CTDL, CTDM, and CTDH groups in a dose-dependent manner in rat liver. And the Caspase-8 protein expression was increased in the CTDM and CTDH groups, suggesting that CTD could induce caspase-dependent apoptosis to cell death in rat liver.

3.5 Integrative hepatotoxicity mechanism analysis

In the study, ERS, autophagy and apoptosis could be activated in CTD-induced DILI rat. First, ERS can either trigger or suppress autophagy[59-61]. Studies revealed that triptolide, diclofenac, and 3-acetyldeoxynivalenol could induce hepatotoxicity through activating "ATF4-CHOP" ERS pathway, furtherly induced autophagy *in vivo* and *in vitro*[62-64]. Hence, hypothesized that CTD-induced ERS could further activate autophagy. The UPR pathway was activated to induce ERS with CHOP protein overexpressed, then triggered the downstream autophagy pathway. Moreover, sustained ERS could lead to the release of Beclin-1[22]. Overall, these results indicated that excessive ERS could activate autophagy caused by CTD, providing an intrinsic link between CTD-induced ERS and autophagy.

Apoptosis is the downstream process of ERS and autophagy, which could be induced when ERS occurs in cells with transmembrane protein receptors on the ER membrane are activated[65]. Sustained ERS could enhance downstream apoptosis pathway when ATF4 protein synthesizing occurs and enters the nucleus, further upregulate the expression of CHOP and the downstream factor growth arrest and DNA damage to cause apoptosis through inhibiting the expression of Bcl2 apoptosis family-related proteins[66]. Moreover, sustained ERS could enhance Caspase-8 activation, further regulated Caspase-3 leading to cell death[67]. And activation of autophagy could promote apoptosis inducing, which constituted an alternative cell-death process. The release of Beclin-1 could be regulated by Bcl-2 phosphorylation, suggesting that autophagy and apoptosis may be triggered to induce cell death by common upstream signals of ERS[54]. In brief, ER protein GRP78 was triggered first after CTD intervention in rat liver, further promoting the downstream ATF4 and CHOP protein expression underlying sustained ERS pathway. Subsequently, autophagy and apoptosis were activated after ERS activation with LC3 and Beclin-1 overexpressed. Therefore, Bcl-2 was inhibited to cause apoptosis. Death receptor capspase-8 was initially triggered to regulate capspase-3 and Bax activating, leading to liver cell death(Fig. 7).

However, the deeply crosstalk role of ERS between autophagy and apoptosis was not clear, and the novel therapy targets action of GRP78, ATF4, Beclin-1 should be verified in the future, which should be furtherly elucidated from a novel insight in CTD-induced DILI mechanism in rat. This investigation implied that inhibiting ERS and autophagy may represent a promising therapeutic strategy to reduce apoptosis for alleviating CTD-induced liver injury.

4 Conclusion

The present study indicated CTD could cause DILI in rats. And the mechanistically studies suggest that "ATF4-CHOP"-dependent ERS and autophagy were activated by CTD, and synergistically inducing apoptosis in rats. However, the crosstalk relationship of ERS and autophagy should be verified the further experiments both *in vivo* and *in vitro*. This study providing a basis for therapy target of ERS from a novel aspect to mitigate the hepatotoxicity in CTD clinically, which has huge prospects for application in reducing hepatotoxicity for further strengthening the applications of CTD and its preparation.

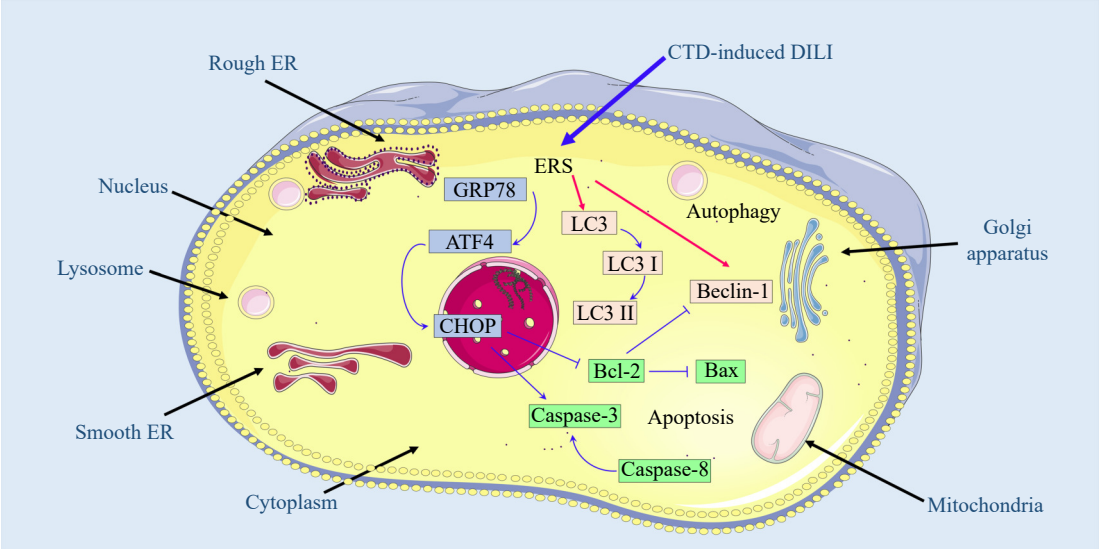

Fig. 7 Potential hepatoxicity mechanism of cantharidin
图 7 斑蝥素潜在肝毒性机制

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