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Protective Effect of Vitamin C on Triptolide-induced Acute Hepatotoxicity in Mice through mitigation of oxidative stress

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Abstract: Triptolide, a purified diterpenoid from the herb *Tripterygium wilfordii* Hook.f., was widely used to treat many diseases. However, the hepatotoxicity of triptolide limited its clinical use. Research showed oxidative stress played an important role in triptolide-induced liver injury. To investigate the effect of vitamin C, which was one of the most effective antioxidants, on triptolide-induced hepatotoxicity and its potential mechanism in mice. In the present study, acute liver injury was induced by intraperitoneal injection of triptolide and vitamin C was orally administered. The results showed treatment with vitamin C prevented the triptolide-induced liver injury by reducing the levels of aspartate transaminase from 286.86 to 192.48 U/mL and alanine aminotransferase from 746.75 to 203.36 U/mL. Histopathological changes of liver corresponded to the same trend. Furthermore, vitamin C also protected the liver against triptolide-induced oxidative stress by inhibiting the generation of malondialdehyde (2.22 to 1.49 nmol/mgprot) and hydrogen peroxide (14.74 to 7.19 mmol/gprot) and restoring the level of total superoxide dismutase (24.32 to 42.55 U/mgprot) and glutathione (7.69 to 13.03 μg/mgprot). These results indicated that vitamin C could protect against triptolide-induced liver injury via reducing oxidative stress, and vitamin C may pose a significant health protection in the clinical use of triptolide.

Key words: vitamin C, triptolide, liver, oxidative stress, mice.

INTRODUCTION

Tripterygium wilfordii Hook.f. (TWHF) was a representative Traditional Chinese Medicine herb (Tao and Lipsky 2000), which had been used in treating autoimmune diseases for centuries, such as nephritis, lupus erythematosus and rheumatoid arthritis (Li et al. 2011, Lin et al. 2007). Triptolide was the major active compound in TWHF (Chen et al. 2018, Wang et al. 2016), which had been shown to have a broad spectrum of biological profiles including anti-inflammatory, immunosuppressive, anti-fertility, anti-cancer activity, neurotrophic and neuroprotective effects (Kong et al. 2018, Xu et al. 2015, 2016, Zheng et al. 2013). However, the widespread use of triptolide raised questions on the safety of its use in clinical settings. More and more

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available data revealed that triptolide exposure results in injury of various organs, including the livers, kidneys, testes, ovaries and hearts (Xi et al. 2017). It affected cells and tissues *in vitro* and *in vivo*. These severe adverse effects limited clinical applications of triptolide. Among the toxicity of triptolide, high incidence of hepatotoxicity was considered as a main cause of triptolide-induced mortality (Jin et al. 2015, Kong et al. 2015, Tan et al. 2018, Wang et al. 2018). The liver was the primary target of reactivation and biotransformation of the drug. Xu et al. showed that liver injury was the main cause of triptolide-induced death by acute and subacute toxicity studies (Xu et al. 2013). Meanwhile, triptolide could impact the expression of functional genes in injured liver (Chen et al. 2007) in addition to triptolide-induced cytotoxicity in human normal liver cells *in vitro* (Yao et al. 2008). Thus, the prevention and elimination of hepatotoxicity induced by triptolide received increasing attention (Hou et al. 2018, Li et al. 2014).

Numerous amounts of research have suggested that oxidative stress was a recognized mechanism for triptolide-induced hepatotoxicity (Xu et al. 2013, Zhang et al. 2016). Triptolide exposure could increase the generation of superoxide anion and inhibit the activity of antioxidant enzymes, and induce oxidative stress in the liver (Li et al. 2014, Xi et al. 2017). For example, triptolide decreased the hepatic glutathione (GSH) level in rats (Yang et al. 2012). It caused a significant reduction in the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) in mice (Li et al. 2014). Furthermore, recent research has shown that triptolide reduced the levels of SOD and GSH and increased the intracellular reactive oxygen species in L-02 and HepG2 cells (Zhou et al. 2017a). Therefore, therapeutic effort for attenuating the oxidative stress could be beneficial in triptolide-induced liver injury.

As we all know, vitamin C, also called ascorbate, is one of the most effective antioxidants. Because of its excellent water solubility, vitamin C could function both intracellularly and extracellularly. Vitamin C could suppress oxidative stress, and its side effects were extremely small. Due to the effectiveness and safety, vitamin C was widely used in the treatment of various diseases. Vitamin C could prevent DNA mutation induced by oxidative stress (Lutsenko et al. 2002), and exert significant protection by reducing reactive oxygen species and renal oxidative damage via its antioxidant activity in maintaining hydroxylase (Dennis and Witting 2017). Published data indicates that the antioxidant effect of vitamin C could protect tissue damage following tunica albuginea incision with tunica vaginalis flap coverage for testicular torsion (Moghimian et al. 2017), thus reminding us that vitamin C had the potential protective action on triptolide-induced hepatotoxicity. In the present study, the aim was to investigate the preventive effect of antioxidant vitamin C on triptolide-induced hepatotoxicity, thus providing more information for the elimination of the clinical toxicity of triptolide.

MATERIALS AND METHODS

MATERIALS

Vitamin C and triptolide were purchased from Sigma Chemical Co., MO, USA. The reagent kit for measurement of total superoxide dismutase (T-SOD), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), alanine aminotransferase (ALT) and aspartate transaminase (AST) were purchased from Nanjing Jiancheng Institute of Biological Engineering Inc. (Jiangsu, China). The reagent kit for measurement of glutathione (GSH) was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China).

ANIMALS AND DRUG TREATMENTS

All animal procedures were accorded to protocols approved by the Animal Care Committee of the Animal Center at the Chinese Academy of Sciences in Shanghai. Adult male, specific-pathogen free (SPF) C57/BL6 mice (18-22 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Animals were maintained under standard laboratory conditions under artificial 12 hours light/12 hours dark cycle.

Animals were randomly divided into four groups, control group (Control, n=6), triptolide group (TP, n=6), vitamin C+triptolide group (VC+TP, n=6), and vitamin C group (VC, n=6). TP and VC+TP groups were treated with the triptolide at 1.0mg/kg (i.p.) (Li et al. 2014). Control and VC groups received i.p. injection of vehicle control (0.9% saline solution containing 1% DMSO). The mice in the VC + TP and VC groups were orally administered with vitamin C (250 mg/kg, the dose relative to human: 27.47mg/kg) 12 and 4 hours before the injection of triptolide or vehicle according to the reference (Jin et al. 2015). Control and TP groups received the vehicle only.

Following injection of triptolide, at 24 hours the eyeball blood was extracted from the mice and they were then sacrificed (Li et al. 2014). The liver was dissected out and collected for further analysis. Liver samples from each mouse was divided into two parts. One part was immersed in 4% paraformaldehyde fixed for hematoxylin-eosin (HE) staining. The other part was homogenized on ice. The concentration of the serum AST and ALT was measured by the blood sample. The level of H_2O_2, T-SOD, GSH and MDA was determined by the supernatant of liver homogenate.

HE STAINING

Liver samples were immersed in 4% paraformaldehyde at 4°C for 24 hours. Then samples received gradient dehydration and were embedded in paraffin for tissue sections. Sections were cut at 5μm (Motic BA410, MOTIC CHINA GROUP CO., LTD) and stained with hematoxylin-eosin according to the standard protocol. Morphologic changes were examined using a light microscope.

MEASUREMENT OF AST AND ALT LEVELS

Assessment of liver function in treated mice was measured with AST and ALT levels. Both of the indexes were determined according to the manufacturer's instruction.

MEASUREMENT OF H_2O_2 PRODUCTION

To determine the effect of vitamin C on oxidative stress in liver injury induced by triptolide, the generation of H_2O_2 level was investigated by assay kit.

MEASUREMENT OF INTRACELLULAR MDA

The supernatant obtained was used for the following determination of intracellular MDA according to the manufacturer's instructions. The date was expressed as opposite OD values by determining the absorbance at 532 nm.

MEASUREMENT OF TOTAL SOD ENZYME ACTIVITY

The level of total SOD (T-SOD) activity in liver was measured using the T-SOD assay kit. The date was expressed as opposite OD values by determining the absorbance at 450 nm.

MEASUREMENT OF GSH LEVEL

GSH levels were measured in different treatment groups by using the assay kit in accordance with the instructions supplied by the manufacturer. The date was expressed as opposite OD values by determining the absorbance at 412 nm.

STATISTICAL ANALYSIS

All of the results were expressed as mean ±S.E.M and analyzed by Origin 9.0 and SPSS 17.0. Statistical analysis was performed using one-way ANOVA followed by a Turkey's multiple range test and $P<0.05$ was considered significant.

RESULTS

HE STAINING

To verify the potential protective effect of vitamin C on triptolide-induced hepatotoxicity *in vivo*, we established an animal model of acute liver injury induced by triptolide. The degree of liver injury was assessed by the histopathological sections and the plasma AST and ALT. For HE staining, pathology observation of liver tissue showed that the Hepatic Lobules structure were clear, liver cells cable were neat and orderly in the control group and vitamin C group (Fig. 1a, d). However, in the triptolide group liver tissue presented the nuclear fragmentation and cytoplasm loosening in hepatic cell, hepatic sinusoid was not distinct, and vascular was congestion (Fig. 1b, f). The severity of histopathological lesions was significantly decreased in the vitamin C + triptolide-treated group (Fig. 1c, g), which indicated no significant differences compared with the control group.

EFFECT OF VITAMIN C ON LIVER INJURY INDUCED BY TRIPTOLIDE IN MICE

As shown in Fig. 2 in the triptolide group, triptolide significantly increased AST and ALT level ($P<0.01$), especially the level of ALT increased nearly three-fold. Furthermore, vitamin C could decrease triptolide-induced increase of serum AST and ALT ($P<0.01$), which indicated that vitamin C has a protective effect on triptolide-induced acute liver injury.

MEASUREMENT OF H_2O_2 GENERATION

Oxidative stress was responsible for cell damage during the progression of hepatotoxicity. To determine the effect of vitamin C on oxidative stress in the liver injury induced by triptolide, H_2O_2 was measured using the test kit. As shown in Fig. 3a, after treatment with triptolide, the H_2O_2 level significantly increased ($P<0.01$). Furthermore, with vitamin C, there was a significant fall as compared with the triptolide-treated ($P<0.01$) from 14.74 to 7.19 mmol/gprot. These data suggested that vitamin C decreased H_2O_2 generation in liver injury induced by triptolide. There were no significant differences in H_2O_2 level between control and VC groups.

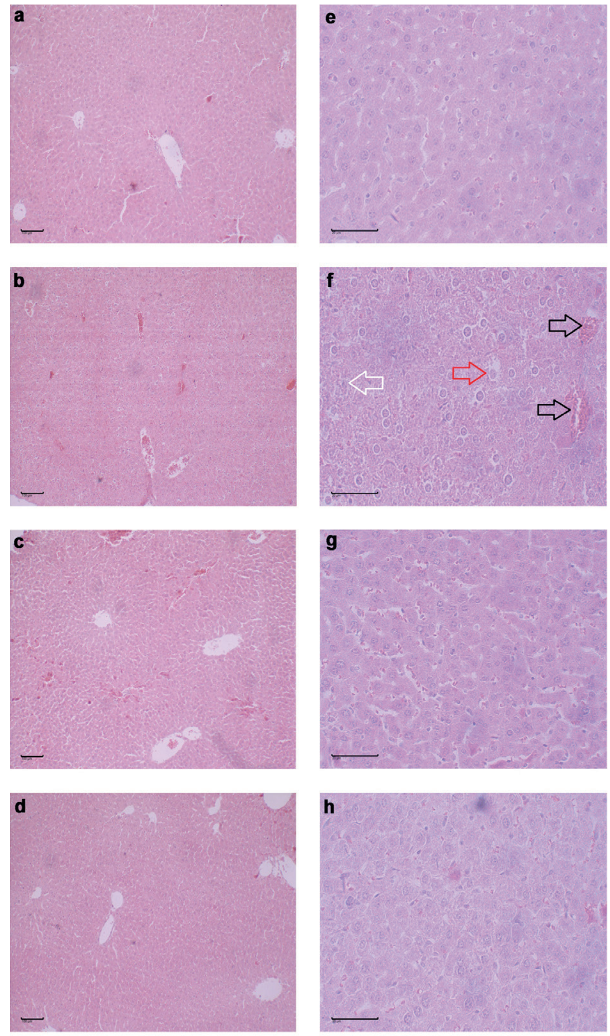

Figure 1 - The HE staining showed liver cellular morphology of each group. n=6/group. The control group (i.p. injection of vehicle control) images were shown in (**a**, **e**). TP group (triptolide group orally administered with vehicle + i.p. injection of triptolide) were shown in (**b**, **f**). VC+ TP group (vitamin C + triptolide group, orally administered with vitamin C+ i.p. injection of triptolide) were shown in (**c**, **g**). VC group (vitamin C group, orally administered with vitamin C+ i.p. injection of vehicle control) were shown in (**d**, **h**). The white arrow showed the nuclear fragmentation. The red arrow showed cytoplasm loosening in hepatic cell. The black arrow showed the congestion of vascular (**f**). Scale bar (**a-d**) =100 µm. Scale bar (**e-h**) =20 µm.

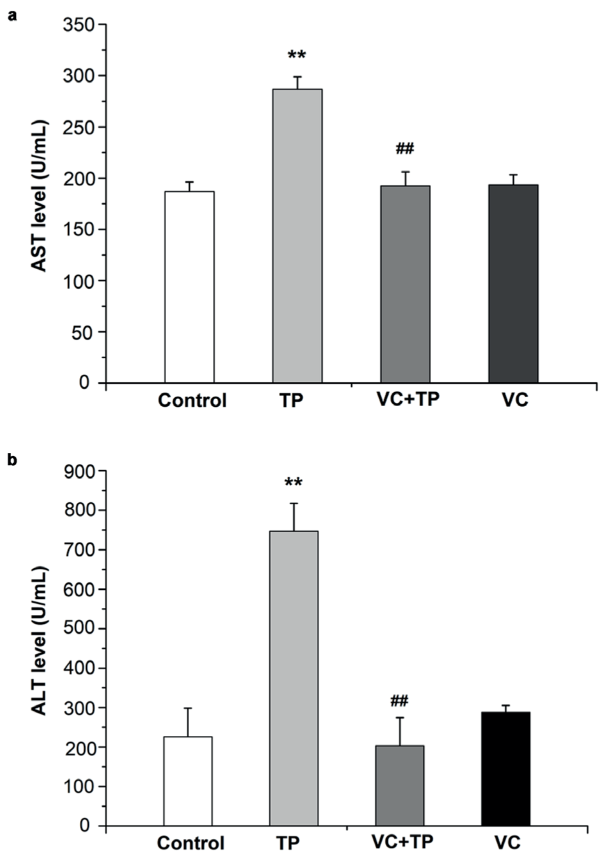

Figure 2 - AST and ALT levels were detected using blood samples. The serum AST level in mice was shown in (**a**). The serum ALT level in mice was shown in (**b**). Data were presented as mean ±S.E.M. n=6/group. $^{**}P<0.01$ vs. Control group. $^{\#\#}P<0.01$ *vs*. TP group. Control group (i.p. injection of vehicle control); TP group (orally administered with vehicle + i.p. injection of triptolide); VC+ TP group (vitamin C + triptolide group, orally administered with vitamin C+ i.p. injection of triptolide); VC group (vitamin C group, orally administered with vitamin C+ i.p. injection of vehicle control).

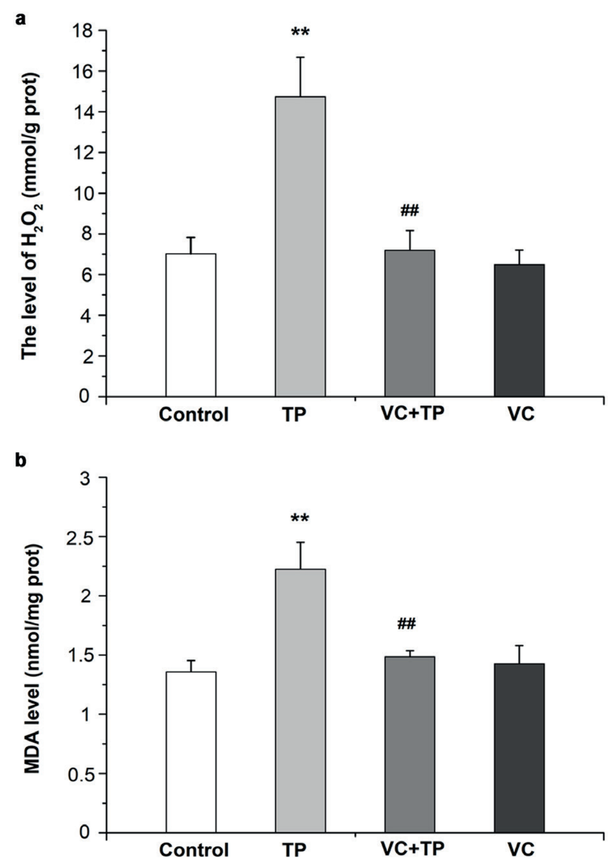

Figure 3 - H_2O_2 level and MDA level were measured in liver lysate. The H_2O_2 level was shown in (**a**). The MDA level was shown in (**b**). Data were presented as mean ±S.E.M. n=6/group. $^{**}P<0.01$ *vs*. Control group. $^{\#\#}P<0.01$ vs. TP group. Control group (i.p. injection of vehicle control); TP group (orally administered with vehicle + i.p. injection of triptolide); VC+ TP group (vitamin C + triptolide group, orally administered with vitamin C+ i.p. injection of triptolide); VC group (vitamin C group, orally administered with vitamin C+ i.p. injection of vehicle control).

MEASUREMENT OF MDA PRODUCTION

MDA was used as a convenient index for determining the extent of lipid peroxidation reactions. The lipid compositions of cells underwent peroxidation during the process of oxidative stress. The results revealed that treatment by triptolide could increase the MDA level in liver ($P<0.05$), which was shown in Fig. 3b. Then, the level of MDA was significantly decreased (from 2.22 to 1.49 nmol/mgprot) in the vitamin C + triptolide treated group ($P<0.05$). There were no significant differences in MDA level between control and VC groups.

MEASUREMENT OF T-SOD ENZYME ACTIVITY

As is well known, SOD could catalyze the free radical species into H_2O. As shown in Fig. 4a, T-SOD activity decreased with treatment of triptolide ($P<0.05$). However, co-treatment with vitamin C enhanced T-SOD level to normal levels ($P<0.05$). These data suggest that triptolide decreased T-SOD activity in liver, and vitamin C could block it. There were no significant differences in T-SOD activity between control and VC groups.

MEASUREMENT OF GSH LEVEL

As main index of antioxidant activitie *in vivo*, the value of GSH can effectively reflect the degree of cell injury in organisms. The results showed that the GSH level determined after 24h exposure to triptolide was 7.69 μg/mg prot. And incubation with vitamin C was markedly increased the level of GSH (P<0.05). There were no significant differences in GSH level between control and VC groups (Fig. 4b).

Taken together, treatment with vitamin C alone did not affect the levels of AST, ALT, H_2O_2, MDA, T-SOD and GSH. These results indicated that vitamin C was safe to the animals.

DISCUSSION

Recently, triptolide had been used widely in clinic, and the incidence of hepatotoxicity was also increasing, which had attracted more and more attention in the world (Wang et al. 2018, Yu et al. 2016, Zhou et al. 2017a). The toxicity of triptolide was usually manifested in acute hepatic necrosis. Triptolide often induced liver damage with hepatomegaly, and increased the level of AST and ALT (Li et al. 2014). In this experiment, histological findings confirmed that there were more necrotic hepatocytes in the triptolide treated only group compared with the control group (Fig. 1). Meanwhile, ALT and AST serum activity in triptolide-treated mice were significantly increased (Fig. 2). These were consistent with the available data, and also confirmed that the acute hepatotoxicity model was established successfully.

At present, the mechanism of hepatotoxicity induced by triptolide is complex (Chen et al. 2018, Xi et al. 2017). The liver is a major site for the transformation of exogenous peroxides into the body. A variety of free radicals are produced in liver cells after the oxidation-reduction, and these free radicals could cause oxidative stress damage in the liver (Zhou et al. 2017b). Oxidative stress has been recognized as an important factor that contributes to drug-induced toxicity (Yang et al. 2012). Previous studies have reported that the hepatotoxicity induced by triptolide is related to the oxidative stress pathway (Jin et al. 2015). Inside, H_2O_2 is closely associated with oxidative stress. GSH is an endogenous antioxidant, which prevents damage to the cellular components by ROS and peroxides. SOD, as one of enzymatic scavengers of free radicals, could limit oxidative injury. It is crucial to maintain the balance between ROS and antioxidant enzymes, which serves as a major mechanism in preventing damage elicited by oxidative stress. A decrease in SOD and GSH could correspond with an increase in cell death, and lipid peroxidation products were generally considered to reflect the cell injury (Deres et al. 2005, Xu et al. 2012). The final concentrations of H_2O_2 and SOD in liver are dependent on the balance between the rate of H_2O_2 generation and its degradation (oxidant-antioxidant balance). The oxidant arm of this balance is formed mainly by H_2O_2 and MDA, and the antioxidant arm is formed by SOD and GSH. But the scavenging capacity of T-SOD or GSH is limited, disturbance of the oxidant-antioxidant balance may affect finally cell function. As our results show, the generation of H_2O_2 and MDA were increased and the T-SOD activity and GSH level decreased significantly in the triptolide-treated mice than in control group (Figs. 3 and 4). The data confirmed that triptolide induced liver cell damage by oxidative stress.

Vitamin C is a common drug in clinical practice. As an important antioxidant, it directly participated in the process of oxidation-reduction reactions and hydroxylation reactions in the body (Hadzi-Petrushev et al. 2017). Vitamin C is purported to eliminate free-radicals, improve immunity. Increasing amounts of evidence show that it is

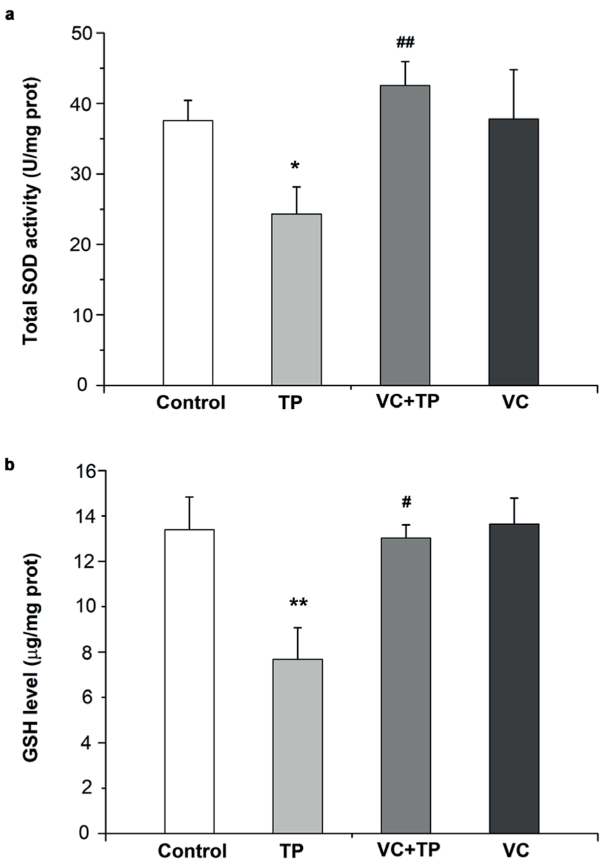

Figure 4 - The total SOD enzyme activity and liver GSH level were measured in liver lysate. The total SOD enzyme activity was shown in (**a**). The liver GSH level was shown in (**b**). Data were presented as mean ±S.E.M. n=6/group. **P*<0.05 *vs*. Control group. ***P*<0.01 *vs*. Control group. #*P*<0.05 *vs.* TP group. ##*P*<0.01 *vs*. TP group. Control group (i.p. injection of vehicle control); TP group (orally administered with vehicle + i.p. injection of triptolide); VC+ TP group (vitamin C + triptolide group, orally administered with vitamin C+ i.p. injection of triptolide); VC group (vitamin C group, orally administered with vitamin C+ i.p. injection of vehicle control).

possible to use vitamin C to treat various diseases, such as paracetamol-induced renal damage (Hadzi-Petrushev et al. 2017), diabetes mellitus (Aguirre-Arias et al. 2017), human tongue carcinoma (Ohwada et al. 2017), kidney dysfunction (Dennis and Witting 2017), hepatic injury (Heidari et al. 2016, Zhong et al. 2017) and so on. However, there remains a lack of information regarding the effect of vitamin C on the acute hepatotoxicity induced by triptolide. Our results show that vitamin C proved to be beneficial in restoring the liver function markers to normal. Compared with the triptolide group, the pathological change and the level of AST and ALT were significantly decreased in the vitamin C group + triptolide (Figs. 1 and 2), which indicates that the liver injury induced by triptolide could be inhibited by vitamin C. It was observed that the venous congestion of the liver had been alleviated in the vitamin C intervention group, the reason might be associated with anti-inflammatory effects by reducing the release of inflammatory mediators and vascular permeability (Chen et al. 2009, Ellulu et al. 2015), or lowering endothelial dysfunction (Barabutis et al. 2017, Wannamethee et al. 2006). The exact mechanism still needs more investigations.

As previously mentioned, the pathological basis of acute liver injury induced by triptolide was oxidative stress, and the study of oxidative stress had become a potential target for the treatment of acute liver injury. The generation of H_2O_2 and MDA could reflect the severity of oxidative stress *in vivo* (Zou et al. 2017). SOD, as the first antioxidant line of body defense, could eliminate the superoxide anion radical (O_2^-) and reflecte the anti-oxidative ability to protect cells from damage. GSH with glutathione peroxidase (GPx) metabolizes hydrogen peroxide and organic hydroperoxodes to hinder the peroxidation chain reaction and protect protein thiol groups from non-enzymatic oxidation (Szaroma et al. 2014). In this experiment, since vitamin C treatment decreased the excess H_2O_2 and MDA in liver cells induced by triptolide treatment in mice (Fig. 3), it was possible that vitamin C scavenging of oxidative stress might have occurred via the endogenous anti-oxidative system, such as induction of SOD and GSH (Miyamoto et al. 2006). Vitamin C, a natural antioxidant, tends to act as a radical scavenger. And it may have some ability to reduce superoxide radical anion that can then be protonated to yield H_2O_2, this activity is less kinetically favourable than dismutation with SOD and GSH. Although it is unclear that how vitamin C upregulates

SOD specifically, increased SOD expression would certainly eliminate superfluous oxygen free radical and protect against oxidative stress damage (Bresciani et al. 2015), then GSH level could be preserved via scavenging superoxide effectively (Fig. 4). In other words, vitamin C protected the liver which was treated with triptolide by increasing the SOD antioxidant potential, and inhibited lipid peroxidation and H_2O_2. The observed effects of vitamin C offered support for its potential use as protective treatment in the toxicity induced by triptolide.

It was noteworthy that although vitamin C inhibited the production of oxidative stress induced by triptolide, the antagonistic effect partially renovated the acute hepatic injury induced by triptolide, all of which suggested that the oxidative damage caused by triptolide might not be the only reason for the hepatotoxicity, there would be other mechanisms leading to the injury, which remain to be further explored.

CONCLUSIONS

In summary, this report demonstrates that vitamin C is able to prevent triptolide-induced liver injury in mice, which could be better for the clinical service, and provide the basis for further research on the safe clinical treatment of triptolide.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: PJX. Performed the experiments: PJX, YYL, RS and ZHY. Analyzed the data: PJX and ZCY. Contributed reagents/materials/analysis tools: PJX and LY. Wrote the paper: PJX.

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