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Comparison of protective effect of ordinary *Cordyceps militaris* and selenium-enriched *Cordyceps militaris* on triptolide-induced acute hepatotoxicity and the potential mechanisms

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ABSTRACT

This study aimed to investigate the prophylactic effect of selenium-enriched *Cordyceps militaris* (CS) against triptolide-induced acute hepatotoxicity. Rats were intragastrically administrated with CS and ordinary *Cordyceps militaris* (CM) for 7 consecutive days prior to triptolide intoxication. Triptolide elevated levels of serum aminotransferases, total cholesterol and caused severe liver injury. However, CS remarkably inhibited the increases of these parameters and pathological damages. The action mechanisms might be associated with its antioxidant property by enhancing antioxidant enzymes activities and triggering nuclear factor E2-related factor 2 (Nrf2) translocation and downstream genes expression. Besides, CS prevented cellular apoptosis through up-regulating Bcl-2/Bax ratio and down-regulating cytochrome *c* and caspase-3 cleavage. Constituent analyses showed that CS had higher amounts of cordycepin, selenium, selenocystine and polysaccharides than CM. In conclusion, CS effectively prevented triptolide-induced hepatotoxicity via activating Nrf2 pathway and inhibiting hepatocyte apoptosis, suggesting that it could be a promising hepatic protector and a befitting nutraceutical supplement.

1. Introduction

Cordyceps militaris is cultured *Cordyceps sinensis* mycelium which is a kind of edible mushrooms and a traditional Chinese medicine widely used in many Asian countries including China, Japan, and Korea (Ng & Wang, 2005). In China, *C. militaris* is highly accepted as functional and medicinal food with tonic effect on the liver. Its active constituents including cordycepin (Lei et al., 2018), polysaccharide (Wu et al., 2017), superoxide dismutase (Coudriet et al., 2017) and microelement (Chan, Barseghyan, Asatiani, & Wasser, 2015) have been reported to contribute to its hepatoprotective activity. Therefore, it has been authenticated as one of New Resources Food approved by Ministry of Public Health in China (Announcement No.3, 2009). It has been reported that *C. militaris* possessed multiple bioactivities, such as anti-inflammation (Choi et al., 2014; Won & Park, 2005), antioxidation (Zhan, Dong, & Yao, 2006), anti-aging (Ji et al., 2009), anti-tumor (Liu, Yang, Yang, Chen, & Li, 1997) and anti-proliferation (Nan et al., 2001). Attributed to those versatile properties, it is usually utilized as an alternative medicine for improving immunity after severe illness (Wang, Meng et al., 2012), enhancing fatigue resistance (Ji et al., 2009), improving insulin secretion (Choi, Park, Choi, Jun, & Park, 2004) and delaying senescence process (Li, Li, Li, Dou, & Gao, 2010). According to traditional Chinese medicine theory, *C. militaris* could regulate liver function (Wang, Lee, Chen, Yu, & Duh, 2012). Many scientific studies also have proved that *C. militaris* has curative effects on various hepatic diseases like HCV (Ueda et al., 2014), liver fibrosis (Liu & Shen, 2003), non-alcoholic fatty liver (Choi et al., 2014), D-galactosamine (D-gal) or lipopolysaccharide-induced fulminant hepatic failure. In clinical practices, *C. militaris* could also prolong the life expectancy of patients with hepatocellular carcinoma (Niwa et al., 2013).

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Researchers have discovered that selenium (Se) level in the blood has a positive relationship with hepatic health status (Outzen et al., 2015). Selenium is an essential micronutrient in human nutrition due to its crucial physiological functions. Selenium deficiency would lead to several diseases including hepatic dysfunction. Therefore, intaking of Se-enriched foods is believed to be a complementary therapy and recommended to people suffering from the hepatic diseases (Bermingham, Hesketh, Sinclair, Koolaard, & Roy, 2014). Nowadays, increasing endeavours have been devoted to producing Se-enriched products like crops, food supplements and pharmaceuticals which possess more remarkable biological activities than their ordinary counterparts (Ip et al., 2000). Due to the immense commercial value, Se-enriched *C. militaris* has been industrially cultivated at a large scale in order to develop highly valued nutraceuticals and alternative medicines in many Asian countries, especially in China. Taken together, both *C. militaris* (Paterson, 2008) and Se (Ursini & Bindoli, 1987) have proved to have excellent antioxidative activities and beneficial to hepatopaths. Therefore, we hypothesized that Se-enriched *C. militaris* might be a more efficient hepatic-protector than its ordinary counterpart.

As reported by World Health Organization, among all the liver diseases, hepatotoxicity induced by taking medicine ranks the top five causes of death worldwide (Lee, 2003). *Tripterygium wilfordii* Hook.f. (TW), a highly valued Chinese medicinal herb, is commonly used to treat autoimmune and inflammatory diseases, including rheumatoid arthritis, systemic lupus erythematosus and nephritis in clinics with a long history (Qiu & Kao, 2003). The major active component in TW is triptolide (TP), which is also widely applied to cure rheumatic and immunological diseases in clinical practices (Liu, 2011; Zhou, Yang, Ding, Li, & Miao, 2012). Unfortunately, applications of TW and TP are restricted to a large extent by their acute toxicities and inevitable adverse effects, especially on the digestive system (Li, Jiang, & Zhang, 2014). Liver is the paramount peptic in human and vulnerable to extraneous toxins (Teschke, 2014), making it the major target organ of TW and TP poisoning. Hence, the development of potential preventive or therapeutic strategies to counteract TP toxicity would be of significant value.

The pathologic foundation of TP-induced hepatotoxicity is believed to involve oxidative stress (Zhou et al., 2014) and mitochondrial control of cellular apoptosis (Fu et al., 2011). Nrf2 pathway has been reported to play a vital role in the cellular protection against oxidative damage, including TP-induced cytotoxicity in rat kidney cells (Li et al., 2012) and HepG2 cells (Li et al., 2014). Furthermore, activation of Nrf2 pathway has been well documented as a valid solution to prevent the TP-induced hepatotoxicity (Guan, Jin, Li, Zhao, & Huang, 2013). Therefore, strategies to modulate the Nrf2 pathway and attenuate oxidative stress are advised to reduce the side effects of TP.

Pretreatments of antioxidative herbal medicines, nutraceuticals and food supplements with minimal adverse reactions are highly appreciated as alternative and complementary approaches to eliminate hepatotoxicity induced by many toxic agents (Yang et al., 2017). However, there still lack studies on whether *C. militaris* could activate Nrf2 pathway or reduce TP-induced hepatotoxicity through the activation of Nrf2. Therefore, in the present study, we comparatively investigated the potential hepatoprotective effect and mechanisms of Se-enriched *C. militaris* and its ordinary counterpart *C. militaris* against TP-induced liver injury *in vivo*.

We previously investigated the protective effect of silymarin on TP-induced oxidative damage, inflammation and apoptosis since it is a well-acknowledged hepatoprotective agent, and has exhibited protective effects against inflammation, oxidation and apoptosis (Avci et al., 2016). It was found that silymarin pretreatment, particularly at high dose (200 mg/kg), could markedly reduce TP-induced hepatotoxicity in rats (Wang et al., 2018). Therefore, silymarin was employed as the positive drug in this study.

Our result for the first time indicated that Se-enriched *C. militaris* significantly attenuated TP-induced hepatotoxicity through inhibiting mitochondria-mediated apoptosis and strengthening endogenous antioxidative defence via activating Nrf2 pathway. This study provided pioneering evidence that Se-enriched *C. militaris* exhibited a superior hepatoprotective effect to ordinary *C. militaris* in TP-induced liver injury. This was also the first report investigating the hepatoprotective effect of Se-enriched products and *C. militaris* against drug-induced hepatotoxicity on *in vivo* murine models.

2. Materials and methods

2.1. Chemicals and materials

Triptolide (TP), silymarin and cordycepin were obtained from Sigma Chemical Co. (St. Louis, MO, USA), with purities all above 98%. Selenium, selenocystine and selenomethionine standard substances were purchased from J&K Scientific Co. (Beijing, China). Se-enriched *C. militaris* (CS) and ordinary *C. militaris* (CM) powder were provided by Fushan Biotech (Shenzhen, China). Commercial kits used to analyze activities of superoxide dismutase (SOD), glutathione (GSH), glutathione-S-transferase (GST), glutathione peroxidase (GSH-Px), catalase (CAT), and malondialdehyde (MDA) were purchased from Jiancheng Institution of Biotechnology (Nanjing, China). Cytochrome *c* (CytC) ELISA kit was obtained from Beijing Chenglin, Co., Ltd. (Beijing, China). And protein concentration was measured with a Thermo Scientific Pierce BCA kit (Fisher Scientific Ireland Ltd., Ireland). The electrochemiluminescence (ECL) kit, nuclear extract kit, anti-Nrf2 antibody, Histone H3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-Actin, anti-procaspase-3 and anti-cleaved caspase-3 antibodies were provided by Bioworld Technology (USA). Anti-Bcl-2 and anti-Bax antibodies were obtained from Abcam (UK). All chemical reagents used in this study were of analytical grade.

2.2. Determination of selenium content of CS and CM

CS and CM powder (0.2 g) were respectively added into a mix of 5 mL HNO_3 and 1 mL H_2O_2 for digestion with a micro digestion instrument (WX-6000, Yiyao Co., Shanghai, China) (Stilinovic et al., 2014). The operational condition was performed as follows (Tie et al., 2014): heated up for 10 min, and digested at a temperature of 180 °C for 15 min. The digest was diluted with distilled water after cooling down and transferred into 50 mL volumetric flask for use.

The instrument for Se analysis was an atomic absorption spectrometer (AAS) equipped with a graphite furnace for atomization (AA-7000, Shimadzu, Japan). The operational condition was performed as follows: detection wavelength was 196 nm; lamp current was 10 mA; slit width was 0.5 nm; injection volume was 10 μL; gas flow was 4 L/min; ashing temperature was 500 °C; atomization temperature was 2500 °C; 1% $Ni(NO_3)_2$ was applied as matrix modifier; deuterium background correction (Tie et al., 2014; Wang & Hou, 2011). Each sample was analyzed in triplicate. The detection limit was estimated by means of analyzing blank control solution through the same procedure, which was three times the standard deviation. A series of selenium standard solutions (0, 50, 100, 150, 200, 250 μg/L) were used to draw calibration curve.

2.3. Determination of cordycepin, selenocystine and selenomethionine content of CS and CM

CS and CM powder (2 g) were respectively extracted with 30-fold Tris-HCl buffer (pH = 6.8) using a magnetic stirring for 4 h. The extracts were centrifuged at 1000*g* for 10 min to obtain the supernatant for subsequent determinations of seleno-aminoacids.

CS and CM powder (2 g) were respectively extracted with 30-fold distilled water at the temperature of 50 °C for 1 h. The extracts were centrifuged at 1000*g* for 10 min to obtain the supernatant for