

Effects of the Kunlun snow chrysanthemum polysaccharides on acetaminophen-induced oxidative stress, inflammation and apoptosis using animal model

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Abstract: To investigate the preventive effect of Kunlun snow chrysanthemum polysaccharides (KSCP) on acetaminophen (AP) induced liver damage and its possible mechanism. Mice acute liver injury model was established via intraperitoneal injection of AP (300 mg/kg). The biochemical indicators of plasma and liver tissue were tested. The effects of KSCP on the liver index were examined. The liver pathological changes were investigated. The expressions of related protein were detected via Western blotting. In our study, compared with model group, the concentrations and contents of ALT, AST, TNF- α , IL-1 β and MDA were reduced and activities of SOD were increase in H-KSCP (1.2mg/10 g)-pretreated mice ($P<0.01$). The liver index was significantly reduced in H-KSCP-pretreated mice compared with model group (4.89 ± 0.22 vs 7.4 ± 0.66 , $P<0.01$). Liver cellular swelling, degeneration and necrosis relieved, and pathological injury had been improved. Western blotting results showed that the caspase-3 protein level in H-KSCP group was significantly decreased, expression of Bcl-2 protein and Bcl-2/Bax ratio was increased, whereas which of Bax protein was decreased ($P<0.01$). KSCP-pretreated at middle and high doses can prevent against the liver injury, its action mechanism may be related to its anti-inflammatory effects and regulation of apoptosis related proteins expression. Overall, our results showed that KSCP may be an effective preventive agent in preventing acute liver injury.

Keywords: Kunlun snow chrysanthemum polysaccharides, acute liver injury, anti-inflammatory, anti-apoptosis, liver protection.

INTRODUCTION

As we all know, the liver is an important organ, so the diseases and conditions that lead to liver inflammation are related to crucial morbidity and mortality (Lefkowitz *et al.*, 2016). Meanwhile, liver is a significant organ responsible for the metabolism of drugs and toxic chemicals (Ko *et al.*, 2017). Acetaminophen (AP) is the most common hepatotoxic chemical agent used in many experimental models for the investigation of acute liver failure (Zhang *et al.*, 2017). Meanwhile, protective effects of a lot of natural products (monomers and mixtures) against AP induced liver injury have been reported (Williams *et al.*, 2015; Mancano., 2017). In addition, recent some of the documentation regarding AP and therapeutic agents targeting its effects show that drug induced liver injury has become the fifth fatal cause of death all over the world (Krenkel *et al.*, 2014).

Currently, due to Chinese medicinal herbs and their extracts have possessed a number of bio-active components, no pollution and less side effects, which have been widely used to prevent different kinds of liver disease (Sharma *et al.*, 2016; Zhang *et al.*, 2017). More and more researchers has paid attention to plant polysaccharides, because of their extensive biological activities and their potential as novel sources of natural

products and drugs. The capitula of *Coreopsis tinctoria*, is otherwise called Kunlun snow chrysanthemum (KSC, Kun Lun Xue Ju) in Al-Sayed *et al.* (2014). It is widely cultivated as both a tea-like beverage and a medicine for centuries, the whole plant was used by the Uyghur nationality in Xinjiang (China) for the prevention of heart disease, cerebrovascular disease and diabetes in traditional Chinese medicine (Yang *et al.*, 2016; Liang *et al.*, 2017). Recent study have reported that water extracts of KSC have not only confirmed these above effects, but also shown that it exert good anti-oxidant and anti-inflammatory activities (Chen *et al.*, 2016). In addition, our previous studies show that Kunlun snow chrysanthemum polysaccharides (KSCP) have a lot of significant pharmacological activities, especially anti-oxidative and anti-inflammatory effects (Zhao *et al.*, 2017). Meanwhile, anti-oxidative stress effect plays a significant role in reducing acute liver injury (Cheong *et al.*, 2016). Thus, one main preventive mechanism for treatment of liver damage consists of preventing the excessive oxidative stress caused by reactive oxygen species, therefore reduce the deleterious effects of toxic agents and the inflammatory response.

But, a few of research has investigated on the preventive and protective effect of KSCP used in traditional Chinese medicine, such as the anti-oxidant, anti-inflammatory and

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anti-apoptosis and so forth. So far, it is hardly known, without the anti-oxidative effects of KSCP, which has been reported previously. Therefore, our group examined whether KSCP has anti-oxidative stress, anti-inflammatory and anti-apoptosis effects, using the *in vivo* model of AP-induced liver damage, also to underline the possible mechanisms.

MATERIALS AND METHODS

Materials

Raw KSC plants were obtained from the Xinjiang Institute of Materia Medica, Xinjiang, China. These plants were authenticated by Prof. Jiang He of Xinjiang University of Chinese Medicine. Kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), superoxide dismutase (SOD), malondialdehyde (MDA), tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) were purchased from Nanjing Jiancheng Biological Technology, Inc (Nanjing, China). Antibodies of Bcl-2, Bax and β -actin were purchased from Cell Signaling Technology (Beijing, China). All other chemical reagents were analytical grade.

Animals

Kunming mice (body weight 20-25 g, n=48 for each sex), were provided by the Laboratory Center, Xinjiang Medical University. The animals were bred and housed under conventional experimental animal facilities with free access to food and water (26°C, 52% air humidity) for 1 week, before AP treatment. In addition, the protocol was approved by the Animal Experiments of Laboratory Center of Xinjiang Medical University, Animal Ethics Committees: Number: IACUC20170212-0512.

KSCP production

First of all, 10 grams of the medical material powder was mixed with distilled water at a ratio of 1:20 (w/v) in a round-bottom flask. The flask was equipped with a condenser and placed into a water bath for 4h at 100°C. And then, the extract (filtrated) was concentrated via vacuum rotary evaporator (50°C and vacuum) and mixed with 100% ethanol (1:5) for 12h (4°C), then which was washed by 80% ethanol 3 folds. Finally, the content of polysaccharide was detected via the phenol-sulfuric acid assay.

The crude polysaccharide extract solution was purified via 20% of sevag mixture solution (chloroform: butyl alcohol: water = 4:1) to remove free proteins. After oscillation and centrifugation, 4-fold volumes of 100% ethanol were added to it for 12h (4°C). After centrifugation, deproteinized polysaccharide extract solution was collected, and then which was initially frozen at -80°C for 24h. The frozen samples were dried via a laboratory freeze-dryer (FD-1A-50, Shanghai Yu Ming Instrument Co., Ltd, China). Finally, the dry KSCP were weighed and stored in an airtight container until use.

Experimental protocol

Acute liver injury was induced by intraperitoneal injection of AP solution (diluted into saline), and the concentration was 300 mg/kg. All Kunming mice were evenly and randomly divided into six groups (n=16). The groups were categorized as:

- (1) normal control (normal saline);
 - (2) AP treatment (300 mg/kg in saline);
 - (3) AP and 0.3 mg/10 g KSCP pre-treatment (KSCP orally feed);
 - (4) AP and 0.6mg/10g KSCP pre-treatment;
 - (5) AP and 1.2mg/10g KSCP pre-treatment;
 - (6) AP and 1.5mg/10g biphenyl diester pills pre-treatment.
- Each mice in KSCP and positive drug groups was orally fed one time daily for consecutive 7 days before model establishment. After 12 h chemical agent-induced, mice were sacrificed via cervical dislocation. Mice blood and liver tissues were collected and immediately stored at -80 for further analyses.

Blood and tissue samples processing and liver index

The blood samples were taken with removing eyeball. After centrifugation (800 rpm and 4°C for 15 min), serum were collected from whole blood samples. Meanwhile, the liver tissues were cleaned with cold saline (4°C), then dried with filter paper and stored at -80°C. According to the following formula, the liver index was calculated:
Liver index = Liver weight (g)/body weight (g) \times 100%

Biochemical estimation of serum enzymes

According to manufacturer's instructions, serum ALT, AST, MDA and SOD level were investigated. Meanwhile, TNF- α and IL-1 β levels were quantified via an enzyme-linked immunosorbent assay (ELISA) kit, and the concentrations are detected via a spectrophotometer (450 nm).

Histopathology

The liver samples were fixed in 10% neutral formalin for 48 h, and then fixed liver tissues were embedded with paraffin. Finally, samples were sectioned into 5- μ m-thick slices, and then which were dyed by hematoxylin-eosin. Histoathological changes of liver injury districts were observed via microscope. Follow the previous report (Zhao *et al.*, 2017), the degree of portal inflammation and hepatocellular necrosis were assessed semi-quantitatively, and then changes were graded as follows: Grade 0: normal histology; Grade 1: presence of degenerated hepatocytes with only rare foci of necrosis; Grade 2: mild centrilobular necrosis around the central vein, occupying only a part of Rappaport's zone; Grade 3: established necrosis limited to zone; and Grade 4: extensive, confluent centrilobular necrosis involving Rappaport's zone. The microscopic examination was performed in a blind way.

Western blot

Briefly, total protein was separated via SDS-PAGE

(12.5% polyacrylamide), and the gel was blotted onto a polyvinylidene difluoride transfer membrane with a semidry blotter for 30 min at 30 V, then which was blocked for 2h in 5% nonfat milk in TBST buffer. Afterward primary and secondary antibodies were incubated with the membranes. After washes in TBST buffer, the transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection system, and the band intensities were determined via AlphaView SA Software (3.4.0.0, Protein Simple, USA).

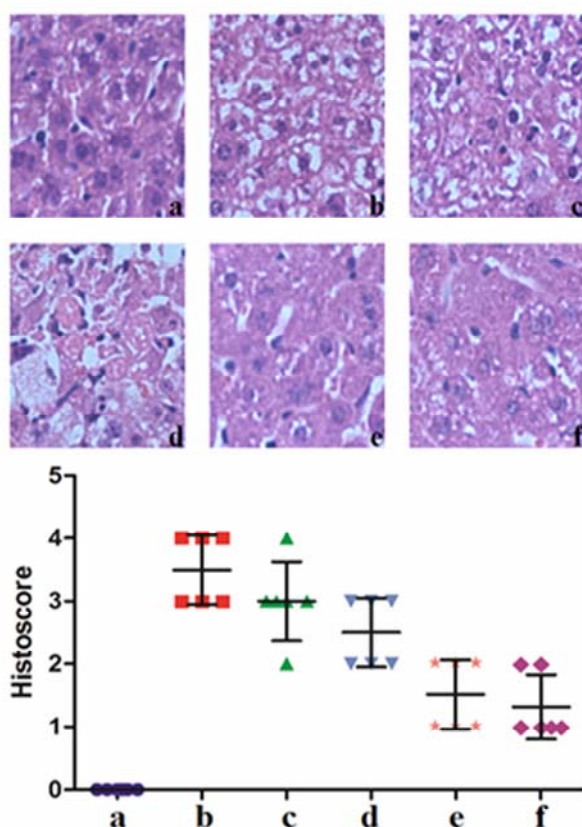


Fig. 1: Histological characterization and effect of KSCP on histopathological scores of livers in the AP-induced liver injury mice (a: Normal, b: Model, c: L-KSCP, d: M-KSCP, e: H-KSCP and f: Positive drug) ($^{##}P<0.01$, $^{*}P<0.05$, $^{**}P<0.01$, n=8).

STATISTICAL ANALYSIS

All the experimental data were expressed as mean \pm standard deviation (SD). T-test and analysis of variance (ANOVA) were used to determine the statistical significance (SPSS version 20.0 software, SPSS Inc., Chicago, USA). A *P* value less than 0.05 or 0.01 was considered significant.

RESULTS

Effect of KSCP on the liver index of mice

Compared with the normal group, the liver index in the model group were significantly increased ($P<0.01$),

meanwhile, there was no significant difference in body weight in the other groups ($P>0.05$). In table 1, compared with the model group, the liver weight and liver index of mice in H-KSCP and biphenyl diester pills groups were obviously reduced ($P<0.01$).

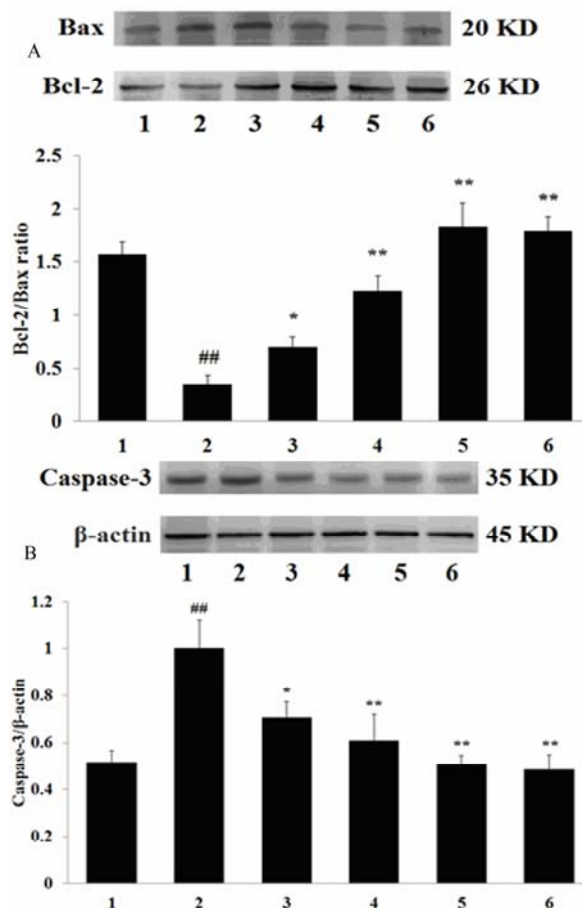


Fig. 2: (A): Bcl-2, Bax and GAPDH expression levels in the liver tissues of each group (1: Normal, 2: Model, 3: L-KSCP, 4: M-KSCP, 5: H-KSCP and 6: Positive drug) ($^{##}P<0.01$, $^{*}P<0.05$, $^{**}P<0.01$, n=8). (B): Caspase-3 and β -actin expression levels in the liver tissues of each group (1: Normal, 2: Model, 3: L-KSCP, 4: M-KSCP, 5: H-KSCP and 6: Positive drug) ($^{##}P<0.01$, $^{*}P<0.05$, $^{**}P<0.01$, n=8).

Hepatic serum levels and antioxidant indexes

Effects of KSCP pre-treatment on liver injury in serum enzyme activities are shown in table 2. Compared with the normal group, these were the significant increase in plasma AST and ALT levels, as follow: ALT was increased to 55.52 ± 4.29 U/L ($P<0.01$) and AST was increased to 177.51 ± 43.82 U/L ($P<0.01$). In addition, these were the same serum levels between H-KSCP and biphenyl diester pills groups ($P>0.05$).

The antioxidant indexes of liver homogenate in every group are listed in table 2. The MDA content in model group was the highest compared with normal group ($P<0.01$). The MDA content in H-KSCP group and biphenyl diester pills group were both the lowest ($P<0.05$).

Table 1: Effects of KSCP on the body weight, liver weight and liver index in the experimental groups (n=8)

| Group | Dose (mg/kg) | Body weight (g) | Liver weight (g) | Liver index (%) |
|---------------|--------------|-----------------|--------------------------|-------------------------|
| Normal | / | 28.33±2.14 | 1.45±0.25 | 5.12±0.58 |
| Model | / | 29.18±2.65 | 2.16±0.47 ^{###} | 7.4±0.66 ^{###} |
| L-KSCP | 0.3 | 28.7±2.82 | 1.84±0.31 | 6.41±0.53 |
| M-KSCP | 0.6 | 28.52±2.59 | 1.68±0.14 [*] | 5.89±0.39 [*] |
| H-KSCP | 1.2 | 29.63±2.51 | 1.45±0.18 ^{**} | 4.89±0.22 ^{**} |
| Positive drug | 1.5 | 28.49±1.72 | 1.36±0.15 ^{**} | 4.77±0.36 ^{**} |

Table 2: Effects of KSCP on ALT, AST and SOD levels and MDA content in the experimental groups (n=8)

| Group | Dose (mg/kg) | ALT (U/L) | AST (U/L) | MDA (nmol/mL) | SOD (U/mL) |
|---------------|--------------|---------------------------|-----------------------------|--------------------------|---------------------------|
| Normal | / | 30.82±1.37 | 52.57±5.22 | 3.81±1.59 | 76.57±15.39 |
| Model | / | 55.52±4.29 ^{###} | 177.51±43.82 ^{###} | 8.47±4.36 ^{###} | 33.28±5.23 ^{###} |
| L-KSCP | 0.3 | 50.24±5.92 | 166.35±29.24 [*] | 7.92±3.03 | 38.61±5.27 |
| M-KSCP | 0.6 | 41.33±5.02 [*] | 83.75±15.41 ^{**} | 5.6±2.37 ^{**} | 63.95±2.84 ^{**} |
| H-KSCP | 1.2 | 32.26±4.61 ^{**} | 56.29±8.5 ^{**} | 3.71±1.71 ^{**} | 80.28±13.58 ^{**} |
| Positive drug | 1.5 | 33.53±2.16 ^{**} | 59.31±6.18 ^{**} | 3.62±1.55 ^{**} | 83.72±9.72 ^{**} |

Table 3: Effects of KSCP on TNF-α and IL-1β levels in the experimental groups (n=8)

| Group | Dose (mg/kg) | TNF-α (pg/mL) | IL-1β (pg/mL) |
|---------------|--------------|-----------------------------|-----------------------------|
| Normal | / | 136.85±33.74 | 179.62±43.26 |
| Model | / | 282.77±58.63 ^{###} | 377.51±62.65 ^{###} |
| L-KSCP | 0.3 | 269.82±64.16 | 350.56±72.36 |
| M-KSCP | 0.6 | 230.62±35.89 [*] | 307.91±52.3 [*] |
| H-KSCP | 1.2 | 150.71±36.92 ^{**} | 191.63±27.32 ^{**} |
| Positive drug | 1.5 | 146.36±22.9 ^{**} | 187.22±24.68 ^{**} |

^{###}*P* < 0.01 vs Normal; ^{*}*P* < 0.05, ^{**}*P* < 0.01 vs Model

The SOD level in model group was the lowest and obviously lower than those in the other group (*P* < 0.05). Compared with the model group, the SOD level in H-KSCP and biphenyl diester pills groups were obviously increased (*P* < 0.01).

Inflammatory cytokines

In table 3, the levels of TNF-α and IL-1β were obviously higher in model group than in normal group (*P* < 0.01). Meanwhile, compared with the model group, H-KSCP and biphenyl diester pills groups significantly declined the production of TNF-α and IL-1β (*P* < 0.01).

Histopathological study

In the results of histopathological study (fig. 1), compared with the normal group, the hepatic cord in the model group was fuzziness and there were a lot of infiltrative inflammatory cells around central vein, and hepatocytes appeared fatty degeneration. In L-KSCP and M-KSCP group, the hepatic lobules were clearer and there were a few infiltrative inflammatory cells. In H-KSCP group and positive group, these was an obvious decline in score (*P* < 0.01), the hepatic lobule was clear, the hepatocytes were unabroken and well-arranged, all structures were closed to that of normal liver.

Western blot analysis

As shown in fig. 2, the results of Western blotting showed that compared with the normal group, the expression level

of the apoptotic protein Bax was significantly increased, while the level of the anti-apoptotic protein Bcl-2 was obviously reduced (*P* < 0.01). By contract, compared with the model group, pretreatment with H-KSCP and Positive drug significantly decreased the injury effects of AP on the levels of both proteins (*P* < 0.01). Additionally, compared with the normal group, model group illustrated a significant increase in the level of caspase-3, while level of caspase-3 was decreased in the H-KSCP group (*P* < 0.01).

DISCUSSION

Liver damage is the pathologic status in the liver system, which can lead to cirrhosis, fibrosis and cancer of the liver (Yang *et al.*, 2017). So to search for effective drugs on prevention and treatment of acute liver injury have already attracted broad concern in the world. Polysaccharides widely exist in plants, microorganisms and animals, and have high efficiency but low toxicity properties (Liang *et al.*, 2017; Liu *et al.*, 2015; Liu *et al.*, 2015). Therefore, polysaccharides had been the hot-spot in the research field of liver-protecting medicines, and had undergone great progresses (Han *et al.*, 2016).

Hepatotoxic agent, AP, is the best-characterized system of xenobiotic-induced hepatotoxicity model for evaluating the hepatoprotective activities of drugs (Bandeira *et al.*,

2017). In this study, when the liver is administrated with AP, the activities of serum ALT and AST in mice are markedly raised, showing that the hepatic tissues were injured, and which are commonly used as sensitive indicators of liver tissue damage (Gao *et al.*, 2017). In addition, oxidative injury induced by AP could be investigated via detecting oxidative stress parameters, such as SOD and MDA and so forth. SOD, a significant endogenous antioxidant enzyme, could markedly decrease free radicals released to remove free radical-initiated lipid per oxidation (Zhao *et al.*, 2017). Therefore, it means that SOD in the body has become an attractive therapeutic strategy of antioxidant supplementation for decreasing the acute liver injury. Besides, the hepatic MDA formation have long been considered as a biomarker of hepatic injury, and which has been the final individual aldehyde resulting from lipid peroxides when lipids are attacked by free radicals, and the raise of MDA has been deemed a significant feature in lesion. These results indicated that pretreatment with H-KSCP could significantly suppressed AP-induced increased of serum ALT and AST and liver index. Meanwhile, it indicated that pretreatment with H-KSCP was able to significantly raise SOD activity and prevent the content of MDA.

Inflammation plays a significant role in AP-induced acute liver injury (Chung *et al.*, 2017). AP-induced hepatic inflammatory response was found to be induced via the action of pro-inflammatory cytokines, such as TNF- α and IL-1 β . These cytokines have been thought to be the focus of investigations of inflammatory organ injury because overproduction of these factors is potentially harmful (Shah *et al.*, 2017). TNF- α not only directly kills hepatocytes, but also promotes liver damage and IL-1 β is associated with the pathogenesis of acute liver injury (Al-Tamimi J *et al.*, 2016). The results of this study show that KSCP may effectively protect the liver by significantly lowering TNF- α and IL-1 β levels and inhibiting the release of inflammatory factors.

Apoptosis, which is widely known as programmed cell death, and can activate a lot of liver diseases (Suda *et al.*, 2016). Therefore, we investigated preventive effects of KSCP on main apoptotic proteins such as Bcl-2, Bax and caspase-3, and which play significant roles in the molecular mechanisms of apoptosis. In our hands, our results showed that pre-administrated of KSCP inhibiting liver cell apoptosis in the mice of acute liver injury. The pro-survival members of the Bcl-2 family such as Bcl-2, it is an effective cell apoptosis inhibitor, and which could significantly inhibit cell apoptosis. Meanwhile, Bcl-2 members are localized in the mitochondria and have either Bcl-2 or pro-apoptotic effector protein Bax. The ratio between the two subsets helps to measure, in part, the susceptibility of cells to an apoptotic signal. In addition, Caspase-3 is a significant active regulator of apoptosis in different cells, involving liver cells (Su *et al.*, 2016). Our results indicated that KSCP pretreatment

reduced hepatocytes apoptotic proteins in dose dependent manner via up regulating the level of Bcl-2 and down regulating the expression of Bax and caspase-3.

CONCLUSION

Overall, the preventive actions of KSCP were investigated via establishing model of acute liver injury, which showed preventive potential of KSCP. The results above, for the first time, indicate that KSCP prevented the acute liver injury through inhibiting lipid per oxidation and oxidative stress, decreasing hepatocyte apoptosis, and suppressing pro-inflammation. Finally, our findings indicate that KSCP may be an effective preventive natural product in preventing acute liver injury. Further studies are necessary to investigate the mechanism by which KSCP is metabolized to protect the liver.

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