Protective Effect of Lycium Barbarum Polysaccharides on Anti-Tuberculosis Drug-Induced Injury to Human Hepatocytes and Its Mechanism

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Abstract

Studies have shown that *Lycium barbarum* polysaccharides (LBPs) have a protective effect on liver injury, but the mechanism is not fully understood. In this work, the effect of LBPs on L-02 cells exposed to anti-tuberculosis drug was investigated and the potential molecular mechanism was explored. Results showed that LBPs significantly prevented anti-tuberculosis drug-induced hepatotoxicity in a dose-dependent manner, as indicated by cell viability and diagnostic indicators of liver injury. The anti-tuberculosis drug promoted the production of reactive oxygen species and enhanced the oxidative stress, as evidenced by an increase in the malondialdehyde level and a decrease in the antioxidant enzyme levels in the liver. These effects were suppressed by treatment with LBPs. Furthermore, exploration of the underlying mechanism of LBPs revealed that the caspase-3 activity was markedly inhibited by the treatment with LBPs in the liver of anti-tuberculosis drug-treated mice. LBPs increased the expression level of Nrf2, thereby inactivating proapoptotic signaling events and restoring the balance between proapoptotic Bax and antiapoptotic Bcl-2 proteins in the cell of anti-tuberculosis drug-treated mice. In conclusion, LBPs inhibited anti-tuberculosis drug-induced apoptosis partly due to its antioxidant and antiapoptosis activities via the Nrf2 signaling pathway.

1. Introduction

Tuberculosis is a chronic infectious disease caused by *Mycobacterium tuberculosis*. Isoniazid (INH) and rifampicin (RFP) are the first line of anti-tuberculosis drugs recommended by the World Health Organization\(^1\). However, INH and RFP have potential liver toxicity, which causes liver function injury in patients. Therefore, prevention and treatment of liver injury caused by anti-tuberculosis drugs has been a research hotspot.

*Lycium barbarum* has been used as a traditional Chinese herbal medicine for thousands of years and has been widely applied as medicine, pharmaceutical ingredient, and functional food in the last 50 years\(^2,3\). *L. barbarum* polysaccharides (LBPs) are the main active substance in *L. barbarum*\(^4\). Increasing evidences show that LBPs can protect the liver from injury induced by hepatotoxins\(^5,6\). However, the activity and mechanism of LBP in protecting liver injury remain unclear and should be further studied.

In this study, an experiment was designed to evaluate the protective ability of LBP against anti-tuberculosis drug-induced liver cell injury and to illustrate the underlying mechanism.

2. Materials And Methods

2.1 Materials and regents

RMPI 1640 medium was purchased from Hyclone (Thermo Fisher, Waltham, USA). Fetal bovine serum (FBS) was purchased from BI (Biological Industries, Beit Haemek, Israel). Reactive oxygen species (ROS) detection kit, lactate dehydrogenase (LDH) kit and mitochondrial membrane potential assay kit were
obtained from Solabio (Beijing, China). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT) as well as glutathione peroxidase (GSH-PX), malondialdehyde (MDA) assay kits was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Annexin V-FITC apoptosis detection kit, phosphatase inhibitor cocktail, nuclear protein and cytoplasmic protein extraction kit was supplied by BestBio (Shanghai, China). All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

The human L-02 liver cell line was obtained from institute of cell biology, Chinese academy sciences (Shanghai, China). LBPs were provided by Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences.

### 2.2 Cell culture

Cells were cultured in RPMI 1640 medium supplemented with 1% penicillin/streptomycin and 10% heat-inactivated FBS in an atmosphere of 5% CO₂ and 90% relative humidity at 37°C.

### 2.3 Cell viability assay

L-02 cells were seeded in 96-well plates at the density of 1.2×10⁵ cells/well in RPMI medium for 24 h, and then treated with INH + RFP. After 4 h exposure, L-02 cells were incubated in the presence or absence of various concentrations LBPs0, LBPs1, LBPs2, LBPs3, LBPs4 or silibinin for 24 h. After incubation, 20 µL MTT (5 mg/mL) was added to each well for further 2 h. Subsequently purple-blue formazan precipitate was dissolved with DMSO (150 µL), and the optical densities (OD) at 495 nm were measured using a microplate reader (DNM-9602G, Perlong, Beijing, China).

### 2.4 Determination of enzyme activities

The cell culture supernatant was harvested and the ATL, AST and LDH levels were determined by commercially available kits according to the manufacturer’s protocols. The supernatants in cell lysates were collected for determination of intracellular activities of MDA, SOD, CAT, and GSH-Px with kits according to the manufacturer’s protocol.

### 2.5 Measurement of ROS accumulation

DCFH-DA (10 µM) was added into each well and incubated for 20 min in dark. Cells were washed in PBS and analyzed by confocal laser scanning microscopy (FV1200, Olympus, Tokyo, Japan).

### 2.6 Cell apoptosis detection

The apoptotic cells were analyzed with the Annexin V-FITC apoptosis detection kit. The cells were digested with trypsin and collected by centrifuging at 2000 rpm for 5 min. After washed with PBS, a total of 400 µL 1×Annexin V Binding Buffer and 5 µL Annexin V-FITC of staining solution was added to the cell suspension. After incubation in the dark at 4 °C for 15 min, 10 µL PI dye were added, mixed and incubated for 5 min. The quantification of apoptosis cells was detected by flow cytometry (LSRFortessa, BD, San Diego, USA).
2.7 Western blot analysis

The cells were scraped and washed with PBS, and homogenised in RIPA buffer with 1% phosphatase inhibitors and 1% PMSF for 25 min on ice. After centrifugation at 12,000 g for 15 min at 4 °C, total protein concentrations of cell extracts were determined by BCA protein assay kit. The cytoplasmic and nuclear proteins were extracted using cytoplasmic and nuclear protein extraction kit according to manufacturer’s protocol. Wes analysis was performed on a Wes system according to the manufacturer’s instructions. In brief, protein samples were diluted to an appropriate concentration in sample buffer, then mixed with Fluorescent Master Mix and heated at 95˚C for 5 min. The samples, blocking reagent, primary antibodies, HRP-conjugated secondary antibodies and chemiluminescent substrate were dispensed into the plate. After plate loading, the separation electrophoresis and immunodetection steps take place in the fully automated capillary system, equipped with Compass for SW analysis software (ProteinSimple, California, USA).

2.11 Statistical analysis

Experimental values were expressed as mean ± standard deviation (SD). Significant difference between groups was performed by one way analysis of variance (ANOVA) by IBM SPSS statistics version 19.0 (IBM SPSS Inc., Chicago, IL, USA). P values less than 0.05 were considered statistically significance.

3. Results

3.1 LBPs could improve the viability of INH and RFP-treated L-02 cells

The viability of L-02 cells and the activities of ALT and AST were shown in figure 1A and 1B. L-02 cells treated with INH and RFP showed growth inhibition in time and dose dependent manner. As shown as figure 1C, LBPs can attenuate the INH and RFP induced injury in L-02 cells and LBPs3 was the most effective component.

3.2 LBPs3 could ameliorate enzyme activity in INH and RFP-treated L-02 cells

As shown in Fig. 2, LBPs3 could ameliorate the increase of ALT, AST, and LDH induced by INH and RFP in L-02 cells. Hence, LBPs3 could reduce the toxicity of INH and RFP on L-02 cells.

3.3 LBPs3 could attenuate oxidative stress in INH and RFP-treated L-02 cells

The ROS level in INH and RFP treated cells had a significant increase and LBPs3 could attenuate this change of ROS level (Fig. 3). Hence, LBPs3 could protect the injury induced by INH and RFP in L-02 cells.

3.4 LBPs3 could restore the activities of antioxidant enzymes in INH and RFP-treated L-02 cells

As shown in Fig. 4, the SOD, CAT, and GSH-Px activities and the GSH content were significantly decreased in INH and RFP-treated cells and treatment with LBPs3 reversed the effects. In additional, LBPs3 could
decrease the accumulation of MDA induced by INH and RFP in L-02 cells.

3.5 LBPs3 could enhance the nuclear translocation of Nrf2 and increase the expression of HO-1, NQO1, and GCLM in INH and RFP-treated L-02 cells

LBPs3 significantly enhanced the nuclear localization of Nrf2 in INH and RFP-treated L-02 cells and the levels of HO-1, NQO1, and GCLM in the LBPs3-treated group increased in a concentration-dependent manner (Fig. 5). So, the induction of antioxidant defense proteins such as HO-1, NQO1, and GCLM by LBPs3-induced Nrf2 translocation may be responsible for hepatoprotection.

3.6 LBPs3 could protect against hepatocyte death in INH and RFP-treated L-02 cells

The results of flow cytometry analysis showed that the percentage of apoptotic L-02 cells treated with INH+RFP significantly increased to 57.74% compared with that in the normal cells (6.30%). LBPs3 could significantly decrease the apoptotic rate of the cells in a dose dependent manner (Fig. 6).

4. Discussion

INH and RFP are first-line drugs used to control tuberculosis (TB). The combination of these drugs (INH/RFP) can potentiate the efficacy of anti-TB therapy and reduce the risk of anti-TB drug resistance. However, the morbidity and severity of hepatotoxicity related to this combination also increased significantly\(^7\). Increasing lines of evidence suggest that these toxic metabolites can induce oxidative stress in the liver\(^8\). In the present work, LBPs3 significantly lowered the INH and RFP-induced increase in the activity of ALT, AST, and LDH and the cell survival rate. Moreover, anti-tuberculosis drugs cause oxidative stress\(^9\). In the present study, LBPs3 reversed the INH and RFP-induced oxidative stress, as evident by the significant elevation of the activities of SOD and GSH-Px and the increase in the concentration of GSH. The levels of MDA markedly decreased, indicating that LBPs3 might inhibit lipid peroxidation and effectively recruit the antioxidative defense system to ameliorate liver injury. Previous studies showed that increasing Nrf-2 activity in hepatic tissues is highly hepatoprotective during chemical-induced oxidative stress by mediating the response of the endogenous antioxidant system\(^10\). The hepatoprotective effect of many naturally occurring compounds is mediated by Nrf-2\(^11\). LBPs3 increased the protein expression of Nrf-2 and its downstream antioxidant enzymes. Hence, LBPs3 played important roles via the Nrf-2 signaling pathway.

The depletion of antioxidant defenses and/or the increase in free radical production deteriorates the prooxidant–antioxidant balance, leading to oxidative stress-induced cell apoptosis\(^12\). LBPs3 could effectively inhibit cell apoptosis in the liver induced by INH and RFP. The results suggested that LBPs3 protected anti-tuberculosis drug-induced liver injury by inhibiting oxidation stress and apoptosis.

5. Conclusion
LBPs3 was regarded as a new and promising agent with high potential in the prevention and treatment of anti-tuberculosis drug-induced liver injury and it protected anti-tuberculosis drug-induced liver injury by inhibiting oxidation stress and apoptosis.

**Declarations**

**Conflict of interest**

None.

**Data availability statement**

All data generated or analysed during this study are included in this published article.

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**Figures**
Figure 1

The effect of crude polysaccharides extracted from Lycium barbarum L. (LBPs) on cell viability in anti-tuberculosis drugs induced L-02 cells. (A) Effect of different concentrations of INH+RFP on hepatocytes viability (n=6); (B) Effect of INH + RFP on ALT and AST activity of hepatocytes (n=3). (C) The effect of LBPs on cell viability in anti-tuberculosis drugs L-02 cells (n=3). Compared with the normal control * p < 0.05, ** p < 0.01; compared with the model control # p < 0.05, ## p < 0.01.
Figure 2

The effect of LBPs3 on the release of ALT, AST and LDH in anti-tuberculosis drugs treated L-02 cells (n=3). (A) ALT activity; (B) AST activity; (C) LDH activity. Compared with the normal control * p < 0.05, ** p < 0.01; compared with the model control # p < 0.05, ## p < 0.01.
Figure 3

The effect of LBPs3 on the level of ROS in anti-tuberculosis drugs treated L-02 cells.
Figure 4

The effect of LBPs3 on the oxidative stress in anti-tuberculosis drugs treated L-02 cells. (n = 3). (A) CAT activity; (B) SOD activity; (C) GSH-Px activity; (D) GSH content; (E) MDA content. Compared with the normal control * p < 0.05, ** p < 0.01; compared with the model control # p < 0.05, ## p < 0.01.
Figure 5

The effect of LBPs3 on expression of Nrf2 pathway-related proteins anti-tuberculosis drugs treated L-02 cells.
The effect of LBPs3 on the cell apoptosis in anti-tuberculosis drugs treated L-02 cells (n=3). (A) Flow scatter plot for apoptosis was detected by flow cytometry; (B) Statistical chart for apoptosis was detected by flow cytometry. Compared with the normal control * p < 0.05, ** p < 0.01; compared with the model control # p < 0.05, ## p < 0.01.