Tanshinone IIA protects against immune-mediated liver injury through activation of t-cell subsets and regulation of cytokines

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Abstract

**Background and Aim:** Tanshinone IIA (TSN) is the major active component of Salvia miltiorrhiza, a traditional Chinese Medicine. TSN protects against antioxidant-induced liver injury, although the exact mechanism is not well understood.

**Materials and Methods:** In this study, the protective effects of TSN was examined by enzyme-linked immunosorbent assay (ELISA) and histochemistry of several cytokines.

**Results:** TSN is found to significantly reduce plasma alanin aminotransferase and aspartate amino trans-ferase levels in mice with concanavalin A-induced immune-mediated liver injury. TSN increases T lymphocyte subset CD3⁺, CD4⁺ and CD8⁺ ratios.

Also, TSN significantly reduces inflammatory cytokines, including interleukin-2, interleukin-4, interferon-gamma and tumor necrosis factor alpha, while elevates anti-inflammatory cytokine, interleukin-10.

**Conclusions:** TSN may provide a potential drug candidate for liver injury therapeutics.

**Keywords:** Tanshinone IIA; liver injury; concanavalin A; cytokines

Introduction

Danshen, the dried stems and roots of *Salvia miltiorrhiza Bunge*, have commonly been used for decades in traditional Chinese medicine for cardio-vascular diseases as a circulating anticoagulant [1,2]. Major pharmacological applications of Danshen include dilating cardiocerebral vessels, suppressing aggregation of platelets, improving circulation, removing blood stasis, protecting against ischemic reperfusion injury, and enhancing the tolerance of ischemic tissue to hypoxia [1,3].

In addition, TSN is reported as an antioxidant [4]. Up to the present, 15 classes of biologically active substrates have been extracted from Danshen [2]. Its active ingredients include diterpenoids and phenolic compounds: Tanshinone IIA (TSN), Tanshinone I, cryptotanshinone, Danshensu etc. Liposoluble TSN can be modified into water-soluble sodium TSN sulfonate (STS), which is used in clinics for coronary heart diseases [2].

Evidence shows that Danshensu, the other phenolic component in Danshen, protects against liver injury and hepatic fibrosis [2,5]. It is intriguing to examine if TSN, which has a structure similar to Danshensu, is protective against liver injury and could be a potential therapeutic drug for clinics.

Liver injury is found in a variety of liver diseases. Immune-mediated liver injury is often observed when autoreactive T-cells destroy hepatocytes or cholangiocytes.
in autoimmune diseases and virus-specific T-cells destroy infected hepatocytes in viral hepatitis [6]. Searching for therapeutic drugs for immune-mediated liver injury with high efficiency and low toxicity has been always challenging [6]. Concanavalin A (ConA)-induced liver injury in animals is a commonly accepted acute immune-mediated liver injury model, which is similar to hepatitis caused by human virus and autoimmune responses [6]. ConA binds to liver cell membrane with high affinity and accumulates in liver after systematic injection. ConA injection in animal livers induces a significant increase of the alanine aminotransferase (ALT) level in plasma within 8–24 hours, and causes lymphocyte invasion into liver tissue, which subsequently leads to liver cell apoptosis and necrosis [7,8].

In ConA-induced immune-mediated liver injury, T-cells and Kupffer cells are activated, which, in turn, upregulates inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interferon-gamma (IFN-γ) [8]. It is reported that antibodies to IFN-γ or TNF-α can completely block ConA-induced liver injury in mice [8].

In the present study, whether TSN, like Danshensu, is possibly protective against liver injury was examined. Our data show that TSN remarkably reduces plasma ALT and AST levels in mice with ConA-induced immune-mediated liver injury. TSN increases T lymphocyte subset CD3⁺, CD4⁺ and CD8⁺ ratios. Also, TSN significantly reduces inflammatory cytokines interleukin-2 (IL-2), interleukin-4 (IL-4), IFN-γ and TNF-α, while elevating anti-inflammatory cytokine interleukin-10 (IL-10) levels.

**Materials and methods**

**Animals and treatments**

Kunming male mice (Chinese People’s Liberation Army Academy of Military Medical Sciences Center, Certificate No. 2007-004, n=60) weighing 18–22 g were caged at room temperature of 22–25°C. The mice were under a 12-h light-dark cycle with free access to food and drinking water. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Mice were randomly divided into 6 groups: normal control group (Normal), ConA group (ConA), Bifendate (Bif) group (ConA+Bif), TSN high (ConA+TSN30), and medium- (ConA+TSN20) and low-dose groups (ConA+TSN10). Immune-mediated liver injury was induced by ConA (Sigma) 20mg kg⁻¹ i.v. via the tail vein injection in mice except the normal control group. The Bif group was given Bif (Beijing Concord) 150mg kg⁻¹ i.g. TSN (Sheng Daw Biomedical Technology Ltd., Shanxi, China) was given at 30, 20 and 10 mg kg⁻¹ i.g. and administered for 15 days. Normal control group mice were given equivalent doses of saline.

After treatments, blood was taken with heparin (Biochemical Pharmaceuticals, Tianjin, China) to measure CD3⁺, CD4⁺ and CD8⁺ by Flow Cytometry and serum ALT, AST, IL-2, IL-4, IL-10, TNF-α and IFN-γ by enzyme-linked immunosorbert assay (ELISA). The right lower liver lobes were fixed with 10% formaldehyde for hematoxylin-eosin (HE) staining.

**Estimations of ALT, AST, CD positive T-cells, IL-2, IFN-γ, IL-4, TNF-α and IL-10 levels in plasma**

Serum ALT and AST were determined by ALT and AST detection ELISA kit as described by the manufacturer (Biosino Bio Technology Co. Ltd., Beijing, China). Plasma CD positive T-cells were measured with a CD positive T-cell kit as described by the manufacturer (BioLegend, Beijing, China). Briefly, blood with anticoagulant (100 μl) was incubated with 1.3 μl Perp-CD3⁺, 0.5 μl FITC-CD4⁺ and 1.3 μl PE-CD8⁺ at room temperature for 30 min in the dark. Erythrocyte (10%, 2ml) was then added at room temperature for 15 min to lyse hemolysin. The mixture was centrifuged and the sediment was washed with phosphate buffered saline (PBS) for three times. Each specimen was examined with 15000 lymphocytes by flow cytometry (CELLQuest). IL-2, IL-4, IL-10, TNF-α and IFN-γ levels in plasma were detected by ELISA kit as described by the manufacturer (Shenzhen Dakota Biology Technology Ltd.).

**Immunohistochemistry**

Mouse liver tissues were fixed with 10% formaldehyde, paraffin-embedded, sliced (5 μm in thickness), and stained with hematoxylin for 5 min. After wash with water and then 1% hydrochloric acid for 30 min, the slices were stained with eosin for 2 min. Sections were then examined by light microscopy. A total of 10 tissue sections were analyzed for each animal.

**Statistical analysis**

Statistical comparisons were made by one-way analysis of variance followed by post hoc two-tail Student’s t test. The statistical significance was set to p < 0.05.

**Results**

**TSN decreases plasma ALT and AST levels in mice with immune-mediated liver injury**

In immune-mediated liver injury animals, the serum AST and ALT levels increase significantly (Figure 1).
ALT and AST levels. Data represent Mean±SD (n = 10).

In TSN20 and TSN30 groups, AST and ALT levels are reduced remarkably compared to liver-injury group (Figure 1). With TSN10 treatment, AST and ALT activities are also suppressed, although there is no statistical significance observed (Figure 1). Our data show that high doses of TSN treatments can effectively reduce the elevation of plasma ALT and AST levels induced by immune-mediated liver injury in mice.

**TSN increases CD positive T-cells in mice with immune-mediated liver injury**

With ConA injection, immune-mediated liver injury induces significant downregulation of CD3⁺, CD4⁺ and CD8⁺ ratios in blood compared with the controls (Figure 2). Treatments of TSN30 and positive control Bif increase CD3⁺, CD4⁺ and CD8⁺ ratios significantly (Figure 2). CD3⁺ and CD8⁺ ratios increases in TSN10 and TSN20 treatment groups. Our data suggest that TSN might reduce T cell cytotoxicity through regulating populations of T lymphocyte subsets.

**TSN regulates cytokine levels in mice with immune-mediated liver injury**

The serum cytokine IL-2, IFN-γ, IL-4, TNF-α and IL-10 significantly increase in liver injury group (Figure 3). TSN30 and Bif treatments remarkably down-regulate all cytokines measured except IL-10, while TSN20 treatment decreases IL-2, IFN-γ and IL-4 levels (Figure 3). In the TSN10 group, only IL-2 and IFN-γ levels are significantly lowered (Figure 3). Different from other cytokines examined in the present experiment, IL-10 is upregulated by TSN10, TSN20 and TSN30 treatments (Figure 3E). Our data indicate that TSN regulates inflammatory and anti-inflammatory cytokines protecting against liver injury.

**Effects of TSN on morphologic changes in liver tissues**

Compared with normal control group (Figure 4A), ConA injection induces invasion of the inflammatory cells around the central vein in the hepatic lobule, serious sinusoidal congestion, substantial liver cell degeneration, edema and liver cell necrosis (Figure 4B). Combination of ConA and Bif induces similar liver tissue pathology with ConA injection alone (Figure 4F). Treatments of TSN30 and Bif reverse all the pathology observed in ConA group (Figure 4C), while treatments with TSN20 and TSN10 still show a small number of sinusoidal congestion, liver cell degeneration and edema, which are much improved compared with ConA treatment group (Figure 4D and 4E).

**Discussion**

Danshen, as a traditional Chinese medicine, has a long history of clinical applications [2]. However, whether the active component TSN of Danshen has any effects on liver injury is not determined yet. This study demonstrated that TSN plays a protective role against ConA-induced immune-mediated liver injury.

Previous studies demonstrate that serum ALT and AST are the most sensitive, specific and commonly used indicators for liver injury evaluation [9]. Our results show that at 12 hours after ConA injection, serum ALT and AST significantly increased (Figure 1), which is consistent with the earlier reports [6,9,10]. These changes correlate with histological pathology including substantial liver cell degeneration, edema and liver cell necrosis (Figure 4A), the pathology also recently reported by others [11]. TSN significantly reduces serum ALT and AST activities as well as histopathology in ConA-induced liver injury indicating...
that TSN is protective against immune-mediated liver injury.

As an immune-mediated liver injury model, ConA injection causes serial dysfunctions of the immune system, including a decrease in blood T-lymphocyte subset CD3+, CD4+ and CD8+ ratios [12]. T-lymphocytes, with the largest cell number and most-complicated cell types, are critical immune effectors and regulators. Peripheral T-cell subsets are accepted as a good indicator for cellular immune status [13].

According to different surface antigens, T-cells can be divided into three subsets: CD3+, CD4+ and CD8+ cells [12,14]. CD3+ cells include all peripheral mature T-cells that represent the overall level of cellular immunity. CD4+ represents T helper lymphocytes (Th) and CD8+ represents inhibit T-lymphocytes [14]. The absolute numbers of CD3+, CD4+, CD8+ and CD4+/CD8+ ratio reflect the cellular immune status [14].

In the present study, TSN is demonstrated to reverse the changes caused by ConA injection in T-lymphocyte subset CD3+, CD4+ and CD8+ ratios, especially at high doses. This suggests that TSN may reduce the consumption of lymphocytes and T-cell invasion into liver during inflammatory responses.

T-cells regulate immune responses through various cytokines. For examples, Th cells, a subgroup of CD4+ cells, secrete cytokine IL-2, IFN-γ, IL-4 and IL-10 [14]. Inflammatory cytokine IL-2, IL-4, IFN-γ and TNF-α

**Figure 3.** TSN regulates cytokine levels in mice with immune-mediated liver injury. Liver injury was estimated by measuring IL-2, IFN-γ, IL-4, TNF-α and IL-10 in plasma by ELISA. (A) Effects of TSN on plasma IFN-γ level. (B) Effects of TSN on plasma TNF-α level. (C) Effects of TSN on plasma IL-2 level. (D) Effects of TSN on plasma IL-4 level. (E) Effects of TSN on plasma IL-10 level. Data represent Mean±SD (n=10). **p<0.01 vs. Normal group, *p<0.05, **p<0.01 vs. ConA group.
are a critical cause of acute liver injury [15]. While anti-inflammatory cytokine IL-10 is protective against immune-mediated liver injury [15]. Inflammatory and anti-inflammatory cytokines interact with each other and keep a mutual balance under physiological conditions.

The present study finds that inflammatory cytokine IL-2, IFN-γ, IL-4, TNF-α and IL-10 increase after ConA injection. TSN, especially with high doses, decreases inflammatory cytokine IL-2, IFN-γ, IL-4 and TNF-α levels, while increasing anti-inflammatory cytokine IL-10, which suggests that TSN reduces inflammatory responses and enhances the protective effects of anti-inflammatory cytokines.

Taken together, our data indicate that TSN provides significant protection against ConA-induced immune-mediated liver injury in mice. TSN protection is mediated by upregulation of T-cell subsets, decrease of inflammatory cytokines and increase of anti-inflammatory cytokine. Our results show that TSN can be a potential drug for immune-mediated liver injury therapy.

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References